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(21) International Application Number: PCT/US98/00153 (22) International Filing Date: 13 January 1998 (13.01.98) (30) Priority Data: 60/035,496 14 January 1997 (14.01.97) US (71) Applicant (for all designated States except US): HUMAN GENOME SCIENCES, INC. [US/US]; 9410 Key West Avenue, Rockville, MD 20850 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): GENTZ, Reiner, L. [DE/US]; 13404 Fairland Park Drive, Silver Spring, MD 20904 (US). NI, Jian [CN/US]; 5502 Manorfield Road, Rockville, MD 20853 (US). EBNER, Reinhard [DE/US]; 9906 Shelbourne Terrace #316, Gaithersburg, MD 20878 (US). YU, Guo-Liang [CN/US]; 13524 Straw Bale Lane, Darnestown, MD 20878 (US). RUBEN, Steven, M. [US/US]; 18528 Heritage Hills Drive, Olney, MD 20832 (US). FENG, Ping [CN/US]; 4 Relda Court, Gaithersburg, MD 20878 (US). (74) Agents: BROOKES, A., Anders et al.; Human Genome Sciences, Inc., 9410 Key West Avenue, Rockville, MD 20850 (US).			(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: TUMOR NECROSIS FACTOR RECEPTORS 6 α AND 6 β			
(57) Abstract The present invention relates to novel Tumor Necrosis Factor Receptor proteins. In particular, isolated nucleic acid molecules are provided encoding the human TNFR- α and -6 β proteins. TNFR-6 α and -6 β polypeptides are also provided as are vectors, host cells and recombinant methods for producing the same. The invention further relates to screening methods for identifying agonists and antagonists of TNFR-6 α and -6 β activity. Also provided are diagnostic methods for detecting immune system-related disorders and therapeutic methods for treating immune system-related disorders.			

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Tumor Necrosis Factor Receptors 6α & 6β

Field of the Invention

The present invention relates to novel human genes encoding polypeptides which are members of the TNF receptor family. More specifically, isolated nucleic acid molecules are provided encoding human polypeptides named tumor necrosis factor receptor- 6α & - 6β hereinafter sometimes referred to as "TNFR- 6α , & - 6β " or generically as "TNFR polypeptides". TNFR polypeptides are also provided, as are vectors, host cells and recombinant methods for producing the same. The invention further relates to screening methods for identifying agonists and antagonists of TNFR polypeptide activity. Also provided are diagnostic and therapeutic methods utilizing such compositions.

Background of the Invention

Many biological actions, for instance, response to certain stimuli and natural biological processes, are controlled by factors, such as cytokines. Many cytokines act through receptors by engaging the receptor and producing an intracellular response.

For example, tumor necrosis factors (TNF) alpha and beta are cytokines which act through TNF receptors to regulate numerous biological processes, including protection against infection and induction of shock and inflammatory disease. The TNF molecules belong to the "TNF-ligand" superfamily, and act together with their receptors or counter-ligands, the "TNF-receptor" superfamily. So far, nine members of the TNF ligand superfamily have been identified and ten members of the TNF-receptor superfamily have been characterized.

Among the ligands there are included TNF- α , lymphotoxin- α (LT- α , also known as TNF- β), LT- β (found in complex heterotrimer LT- α 2- β), FasL, CD40L, CD27L, CD30L, 4-1BBL, OX40L and nerve growth factor (NGF). The superfamily of TNF receptors includes the p55TNF receptor, p75TNF receptor, TNF receptor-related protein, FAS antigen or APO-1, CD40, CD27, CD30, 4-1BB, OX40, low affinity p75 and NGF-receptor (Meager, A., *Biologicals*, 22:291-295 (1994)).

Many members of the TNF-ligand superfamily are expressed by activated T-cells, implying that they are necessary for T-cell interactions with other cell types which underlie cell ontogeny and functions. (Meager, A., supra).

5 Considerable insight into the essential functions of several members of the TNF receptor family has been gained from the identification and creation of mutants that abolish the expression of these proteins. For example, naturally occurring mutations in the FAS antigen and its ligand cause lymphoproliferative disease (Watanabe-Fukunaga, R., et al., Nature 356:314 (1992)), perhaps
10 reflecting a failure of programmed cell death. Mutations of the CD40 ligand cause an X-linked immunodeficiency state characterized by high levels of immunoglobulin M and low levels of immunoglobulin G in plasma, indicating faulty T-cell-dependent B-cell activation (Allen, R.C. et al., Science 259:990 (1993)). Targeted mutations of the low affinity nerve growth factor receptor
15 cause a disorder characterized by faulty sensory innervation of peripheral structures (Lee, K.F. et al., Cell 69:737 (1992)).

 TNF and LT- α are capable of binding to two TNF receptors (the 55- and 75-kd TNF receptors). A large number of biological effects elicited by
20 TNF and LT- α , acting through their receptors, include hemorrhagic necrosis of transplanted tumors, cytotoxicity, a role in endotoxic shock, inflammation, immunoregulation, proliferation and anti-viral responses, as well as protection against the deleterious effects of ionizing radiation. TNF and LT- α are involved
25 in the pathogenesis of a wide range of diseases, including endotoxic shock, cerebral malaria, tumors, autoimmune disease, AIDS and graft-host rejection (Beutler, B. and Von Huffel, C., Science 264:667-668 (1994)). Mutations in the p55 Receptor cause increased susceptibility to microbial infection.

 Moreover, an about 80 amino acid domain near the C-terminus of TNFR1 (p55) and Fas was reported as the "death domain," which is
30 responsible for transducing signals for programmed cell death (Tartaglia et al., Cell 74:845 (1993)).

 Apoptosis, or programmed cell death, is a physiologic process essential to the normal development and homeostasis of multicellular organisms (H. Steller, Science 267, 1445-1449 (1995)). Derangements of apoptosis contribute to the pathogenesis of several human diseases including cancer,
35 neurodegenerative disorders, and acquired immune deficiency syndrome (C.B. Thompson, Science 267, 1456-1462 (1995)). Recently, much attention has focused on the signal transduction and biological function of two cell surface death receptors, Fas/APO-1 and TNFR-1 (J.L. Cleveland, J.N. Ihle, Cell 81,

479-482 (1995); A. Fraser, G. Evan, *Cell* 85, 781-784 (1996); S. Nagata, P. Golstein, *Science* 267, 1449-56 (1995)). Both are members of the TNF receptor family which also include TNFR-2, low affinity NGFR, CD40, and CD30, among others (C.A. Smith, et al., *Science* 248, 1019-23 (1990); M. Tewari, V.M. Dixit, in *Modular Texts in Molecular and Cell Biology* M. Purton, Heldin, Carl, Ed. (Chapman and Hall, London, 1995). While family members are defined by the presence of cysteine-rich repeats in their extracellular domains, Fas/APO-1 and TNFR-1 also share a region of intracellular homology, appropriately designated the "death domain", which is distantly related to the *Drosophila* suicide gene, reaper (P. Golstein, D. Marguet, V. Depraetere, *Cell* 81, 185-6 (1995); K. White et al., *Science* 264, 677-83 (1994)). This shared death domain suggests that both receptors interact with a related set of signal transducing molecules that, until recently, remained unidentified. Activation of Fas/APO-1 recruits the death domain-containing adapter molecule FADD/MORT1 (A.M. Chinnaiyan, K. O'Rourke, M. Tewari, V. M. Dixit, *Cell* 81, 505-12 (1995); M. P. Boldin, et al., *J. Biol Chem* 270, 7795-8 (1995); F.C. Kischkel, et al., *EMBO* 14, 5579-5588 (1995)), which in turn binds and presumably activates FLICE/MACH1, a member of the ICE/CED-3 family of pro-apoptotic proteases (M. Muzio et al., *Cell* 85, 817-827 (1996); M.P. Boldin, T.M. Goncharov, Y.V. Goltsev, D. Wallach, *Cell* 85, 803-815 (1996)). While the central role of Fas/APO-1 is to trigger cell death, TNFR-1 can signal an array of diverse biological activities-many of which stem from its ability to activate NF-kB (L.A. Tartaglia, D.V. Goeddel, *Immunol Today* 13, 151-3 (1992)). Accordingly, TNFR-1 recruits the multivalent adapter molecule TRADD, which like FADD, also contains a death domain (H. Hsu, J. Xiong, D.V. Goeddel, *Cell* 81, 495-504 (1995); H. Hsu, H.-B. Shu, M.-P. Pan, D.V. Goeddel, *Cell* 84, 299-308 (1996)). Through its associations with a number of signaling molecules including FADD, TRAF2, and RIP, TRADD can signal both apoptosis and NF-kB activation (H. Hsu, H.-B. Shu, M.-P. Pan, D.V. Goeddel, *Cell* 84, 299-308 (1996); H. Hsu, J. Huang, H.-B. Shu, V. Baichwal, D.V. Goeddel, *Immunity* 4, 387-396 (1996)).

The effects of TNF family ligands and TNF family receptors are varied and influence numerous functions, both normal and abnormal, in the biological processes of the mammalian system. There is a clear need, therefore, for identification and characterization of such receptors and ligands that influence biological activity, both normally and in disease states. In particular, there is a need to isolate and characterize novel members of the TNF receptor family.

Summary of the Invention

The present invention provides isolated nucleic acid molecules comprising a polynucleotide encoding at least a portion of a TNFR-6 α or -6 β polypeptide having the complete amino acid sequences shown in SEQ ID NOS:2 and 4, respectively, or the complete amino acid sequence encoded by a cDNA clone deposited as plasmid DNA as ATCC Deposit Number 97810 and 97809, respectively. The nucleotide sequence determined by sequencing the deposited TNFR-6 α and -6 β clones, which are shown in Figures 1 and 2 (SEQ ID NOS:1 and 3, respectively), contain open reading frames encoding complete polypeptides of 300 and 170 amino acid residues, respectively, including an initiation codon encoding an N-terminal methionine at nucleotide positions 25-27 and 73-75 in SEQ ID NOS: 1 and 3, respectively.

The TNFR proteins of the present invention share sequence homology with other TNF receptors. Splice variants TNFR-6 α and -6 β show the highest degree of sequence homology with the translation products of the human mRNAs for TNFR-I and -II (Figure 3) (SEQ ID NOS:5 and 6, respectively) also including multiple conserved cysteine rich domains.

The TNFR-6 α and -6 β polypeptides have predicted leader sequences of 30 amino acids each; and the amino acid sequence of the predicted mature TNFR-6 α and -6 β polypeptides are also shown in Figures 1 and 2 as amino acid residues 31-300 (SEQ ID NO:2) and 31-170 (SEQ ID NO:4), respectively.

Thus, one aspect of the invention provides an isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence selected from the group consisting of: (a) a nucleotide sequence encoding a TNFR polypeptide having the complete amino acid sequence in SEQ ID NO:2 or 4, or as encoded by the cDNA clone contained in ATCC Deposit No. 97810 or

97809; (b) a nucleotide sequence encoding a mature TNFR polypeptide having the amino acid sequence at positions 31-300 in SEQ ID NO:2, or 31-170 in SEQ ID NO:4, or as encoded by the cDNA clone contained in ATCC Deposit No. 97810 or 97809; (c) a nucleotide sequence encoding a soluble extracellular domain of a TNFR polypeptide having the amino acid sequence at positions 31 to 283 in SEQ ID NO:2 or 31 to 166 in SEQ ID NO:4, or as encoded by the cDNA clone contained in the ATCC Deposit No. 97810 or 97809; and (d) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), or (c) above.

Further embodiments of the invention include isolated nucleic acid molecules that comprise a polynucleotide having a nucleotide sequence at least 90% identical, and more preferably at least 95%, 96%, 97%, 98% or 99% identical, to any of the nucleotide sequences in (a), (b), (c) and (d) above, or a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide in (a), (b), (c) or (d), above. This polynucleotide which hybridizes does not hybridize under stringent hybridization conditions to a polynucleotide having a nucleotide sequence consisting of only A residues or of only T residues. An additional nucleic acid embodiment of the invention relates to an isolated nucleic acid molecule comprising a polynucleotide which encodes the amino acid sequence of an epitope-bearing portion of a TNFR polypeptide having an amino acid sequence in (a), (b) or (c), above.

The present invention also relates to recombinant vectors, which include the isolated nucleic acid molecules of the present invention, and to host cells containing the recombinant vectors, as well as to methods of making such vectors and host cells and for using them for production of TNFR polypeptides or peptides by recombinant techniques.

The invention further provides an isolated TNFR polypeptide comprising an amino acid sequence selected from the group consisting of: (a) the amino acid sequence of a full-length TNFR polypeptide having the complete amino acid sequence shown in SEQ ID NO:2 or 4 or as encoded by the cDNA clone contained in ATCC Deposit No. 97810 or 97809; (b) the amino acid sequence of a mature TNFR polypeptide having the amino acid sequence at positions 31-300 in SEQ ID NO:2, or 31-170 in SEQ ID NO:4, or as encoded by the cDNA clone contained in ATCC Deposit No. 97810 or 97809; or (c) the amino acid sequence of a soluble extracellular domain of a TNFR polypeptide having the amino acid sequence at positions 31 to 283 in SEQ ID NO:2 or 31 to 166 in SEQ ID NO:4, or as encoded by the cDNA clone contained in ATCC Deposit No. 97810 or 97809.

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The polypeptides of the present invention also include polypeptides having an amino acid sequence at least 80% identical, more preferably at least 90% identical, and still more preferably 95%, 96%, 97%, 98% or 99% identical to those described in (a), (b) or (c) above, as well as polypeptides having an amino acid sequence with at least 90% similarity, and more preferably at least 95% similarity, to those above.

An additional embodiment of this aspect of the invention relates to a peptide or polypeptide which comprises the amino acid sequence of an epitope-bearing portion of a TNFR polypeptide having an amino acid sequence described in (a), (b) or (c), above. Peptides or polypeptides having the amino acid sequence of an epitope-bearing portion of a TNFR polypeptide of the invention include portions of such polypeptides with at least six or seven, preferably at least nine, and more preferably at least about 30 amino acids to about 50 amino acids, although epitope-bearing polypeptides of any length up to and including the entire amino acid sequence of a polypeptide of the invention described above also are included in the invention.

In another embodiment, the invention provides an isolated antibody that binds specifically to a TNFR polypeptide having an amino acid sequence described in (a), (b) or (c) above. The invention further provides methods for isolating antibodies that bind specifically to a TNFR polypeptide having an amino acid sequence as described herein. Such antibodies are useful diagnostically or therapeutically as described below.

Tumor Necrosis Factor (TNF) family ligands are known to be among the most pleiotropic cytokines, inducing a large number of cellular responses, including cytotoxicity, anti-viral activity, immunoregulatory activities, and the transcriptional regulation of several genes. The invention also provides for pharmaceutical compositions comprising TNFR polypeptides, particularly human TNFR polypeptides, which may be employed, for instance, to treat infectious disease including HIV infection, endotoxic shock, cancer, autoimmune diseases, graft vs. host disease, acute graft rejection, chronic graft rejection, neurodegenerative disorders, myelodysplastic syndromes, ischemic injury, toxin-induced liver disease, septic shock, cachexia and anorexia. Methods of treating individuals in need of TNFR polypeptides are also provided.

The invention further provides compositions comprising a TNFR polynucleotide or a TNFR polypeptide for administration to cells *in vitro*, to cells *ex vivo* and to cells *in vivo*, or to a multicellular organism. In certain particularly preferred embodiments of this aspect of the invention, the compositions comprise a TNFR polynucleotide for expression of a TNFR

polypeptide in a host organism for treatment of disease. Particularly preferred in this regard is expression in a human patient for treatment of a dysfunction associated with aberrant endogenous activity of a TNFR polypeptide.

In another aspect, a screening assay for agonists and antagonists is provided which involves determining the effect a candidate compound has on TNFR polypeptide binding to a TNF-family ligand. In particular, the method involves contacting the TNF-family ligand with a TNFR polypeptide and a candidate compound and determining whether TNFR polypeptide binding to the TNF-family ligand is increased or decreased due to the presence of the candidate compound. In this assay, an increase in binding of a TNFR polypeptide over the standard binding indicates that the candidate compound is an agonist of TNFR polypeptide binding activity and a decrease in TNFR polypeptide binding compared to the standard indicates that the compound is an antagonist of TNFR polypeptide binding activity.

TNFR- 6α and - 6β are expressed in endothelial cells, keratinocytes, normal prostate and prostate tumor tissue. For a number of disorders of these tissues or cells, particularly of the immune system, significantly higher or lower levels of TNFR gene expression may be detected in certain tissues (e.g., cancerous tissues) or bodily fluids (e.g., serum, plasma, urine, synovial fluid or spinal fluid) taken from an individual having such a disorder, relative to a "standard" TNFR gene expression level, i.e., the TNFR expression level in healthy tissue from an individual not having the immune system disorder. Thus, the invention provides a diagnostic method useful during diagnosis of such a disorder, which involves: (a) assaying TNFR gene expression level in cells or body fluid of an individual; (b) comparing the TNFR gene expression level with a standard TNFR gene expression level, whereby an increase or decrease in the assayed TNFR gene expression level compared to the standard expression level is indicative of disorder in the immune system.

An additional aspect of the invention is related to a method for treating an individual in need of an increased level of TNFR polypeptide activity in the body comprising administering to such an individual a composition comprising a therapeutically effective amount of an isolated TNFR polypeptide of the invention or an agonist thereof.

A still further aspect of the invention is related to a method for treating an individual in need of a decreased level of TNFR polypeptide activity in the body comprising, administering to such an individual a composition comprising

a therapeutically effective amount of a TNFR antagonist. Preferred antagonists for use in the present invention are TNFR-specific antibodies.

Brief Description of the Figures

Figure 1 shows the nucleotide sequence (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) of TNFR-6 α .

Figure 2 shows the nucleotide sequence (SEQ ID NO:3) and deduced amino acid sequence (SEQ ID NO:4) of TNFR-6 β .

Figure 3 shows an alignment created by the Clustal method using the Megaline program in the DNASTAR suite comparing the amino acid sequences of TNFR-6 α ("TNFR-6a"), and TNFR-6 β ("TNFR-6b") with other TNF receptors, as follows: TNFR1 (SEQ ID NO:5); TNFR2 (SEQ ID NO:6); NGFR (SEQ ID NO:7); LT β R (SEQ ID NO:8); FAS (SEQ ID NO:9); CD27 (SEQ ID NO:10); CD30 (SEQ ID NO:11); CD40 (SEQ ID NO:12); 4-1BB (SEQ ID NO:13); OX40 (SEQ ID NO:14); VC22 (SEQ ID NO:15); and CRMB (SEQ ID NO:16).

Figures 4 and 5 show separate analyses of the TNFR-6 α and -6 β amino acid sequences, respectively. Alpha, beta, turn and coil regions; hydrophilicity and hydrophobicity; amphipathic regions; flexible regions; antigenic index and surface probability are shown. In the "Antigenic Index - Jameson-Wolf" graphs, the indicate location of the highly antigenic regions of the proteins, i.e., regions from which epitope-bearing peptides of the invention may be obtained.

Figure 6 shows the nucleotide sequences of HELDI06R and HCEOW38R which are related to SEQ ID NOS:1 and 3.

Detailed Description

The present invention provides isolated nucleic acid molecules comprising a polynucleotide encoding a TNFR-6 α or -6 β polypeptide, generically "TNFR polypeptide(s)" having the amino acid sequence shown in SEQ ID NOS:2 and 4, respectively, which were determined by sequencing cloned cDNAs. The nucleotide sequences shown in Figures 1 and 2 (SEQ ID NOS:1 and 3) were obtained by sequencing the HPHA52 and HTPCH84 clones, which were deposited on November 22, 1996 at the American Type Culture Collection, 12301 Park Lawn Drive, Rockville, Maryland 20852, and given accession numbers ATCC 97810 and 97809, respectively. The deposited

clones are contained in the pBluescript SK(-) plasmid (Stratagene, La Jolla, CA).

The TNFR-6 α and -6 β proteins of the present invention are splice variants which share an identical nucleotide and amino acid sequence over the N-terminal 142 residues of the respective proteins. The amino acid sequences of these proteins are about 23% similar to and share multiple conserved cysteine rich domains with the translation product of the human TNFR-2 mRNA (Figure 3) (SEQ ID NO:6). Importantly, these proteins share substantial sequence similarity over their extracellular domains including four repeated cysteine rich motifs with significant intersubunit homology. TNFR-2 is thought to exclusively mediate human T-cell proliferation by TNF (PCT WO/94/09137).

Nucleic Acid Molecules

Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA sequencer (such as the Model 373 from Applied Biosystems, Inc., Foster City, CA), and all amino acid sequences of polypeptides encoded by DNA molecules determined herein were predicted by translation of a DNA sequence determined as above. Therefore, as is known in the art for any DNA sequence determined by this automated approach, any nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. The actual sequence can be more precisely determined by other approaches including manual DNA sequencing methods well known in the art. As is also known in the art, a single insertion or deletion in a determined nucleotide sequence compared to the actual sequence will cause a frame shift in translation of the nucleotide sequence such that the predicted amino acid sequence encoded by a determined nucleotide sequence will be completely different from the amino acid sequence actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

By "nucleotide sequence" of a nucleic acid molecule or polynucleotide is intended, for a DNA molecule or polynucleotide, a sequence of deoxyribonucleotides, and for an RNA molecule or polynucleotide, the corresponding sequence of ribonucleotides (A, G, C and U), where each thymidine deoxyribonucleotide (T) in the specified deoxyribonucleotide sequence is replaced by the ribonucleotide uridine (U).

Using the information provided herein, such as the nucleotide sequences in Figures 1 and 2 (SEQ ID NOS:1 and 3), a nucleic acid molecule of the present invention encoding a TNFR polypeptide may be obtained using standard cloning and screening procedures, such as those for cloning cDNAs using mRNA as starting material. Illustrative of the invention, the TNFR-6 α and -6 β clones (Figures 1 and 2, respectively) were identified in cDNA libraries from the following tissues: endothelial cells, keratinocytes, normal prostate tissue, and prostate tumor tissue.

The determined nucleotide sequences of the TNFR cDNAs of Figures 1 and 2 (SEQ ID NOS:1 and 3) contain open reading frames encoding proteins of 300 and 170 amino acid residues, with an initiation codon at nucleotide positions 25-27 and 73-75 of the nucleotide sequences in Figures 1 and 2 (SEQ ID NOS:1 and 3), respectively.

The open reading frames of the TNFR-6 α and -6 β genes share sequence homology with the translation product of the human mRNA for TNFR-2, including the soluble extracellular domain of about residues 31-283 of SEQ ID NO:2 and 31-166 of SEQ ID NO:4, respectively.

As one of ordinary skill would appreciate, due to the possibilities of sequencing errors discussed above, the actual complete TNFR polypeptides encoded by the deposited cDNAs, which comprise about 300 and 170 amino acids, may be somewhat longer or shorter. More generally, the actual open reading frames may be anywhere in the range of ± 20 amino acids, more likely in the range of ± 10 amino acids, of that predicted from the first methionine codon from the N-terminus shown in Figures 1 and 2 (SEQ ID NOS:1 and 3), which is in-frame with the translated sequences shown in each respective figure. It will further be appreciated that, depending on the analytical criteria used for identifying various functional domains, the exact "address" of the extracellular and transmembrane domain(s) of the TNFR polypeptides may differ slightly from the predicted positions above. For example, the exact location of the extracellular domain in SEQ ID NO:2 may vary slightly (e.g., the address may "shift" by about 1 to about 20 residues, more likely about 1 to about 5 residues) depending on the criteria used to define the domain. In any event, as discussed further below, the invention further provides polypeptides having various residues deleted from the N-terminus of the complete polypeptide, including polypeptides lacking one or more amino acids from the N-terminus of the extracellular domain described herein, which constitute soluble forms of the extracellular domains of the TNFR-6 α & -6 β proteins.

Leader and Mature Sequences

The amino acid sequences of the complete TNFR proteins include a leader sequence and a mature protein, as shown in SEQ ID NOS:2 and 4. More in particular, the present invention provides nucleic acid molecules encoding mature forms of the TNFR proteins. Thus, according to the signal hypothesis, once export of the growing protein chain across the rough endoplasmic reticulum has been initiated, proteins secreted by mammalian cells have a signal or secretory leader sequence which is cleaved from the complete polypeptide to produce a secreted "mature" form of the protein. Most mammalian cells and even insect cells cleave secreted proteins with the same specificity. However, in some cases, cleavage of a secreted protein is not entirely uniform, which results in two or more mature species of the protein. Further, it has long been known that the cleavage specificity of a secreted protein is ultimately determined by the primary structure of the complete protein, that is, it is inherent in the amino acid sequence of the polypeptide. Therefore, the present invention provides a nucleotide sequence encoding a mature TNFR polypeptide having the amino acid sequence encoded by a cDNA clone identified as ATCC Deposit No. 97810 or 97809. By the "mature TNFR polypeptides having the amino acid sequence encoded by a cDNA clone in ATCC Deposit No. 97810, or 97809" is meant the mature form(s) of the protein produced by expression in a mammalian cell (e.g., COS cells, as described below) of the complete open reading frame encoded by the human DNA sequence of the clone contained in the deposited vector.

In addition, methods for predicting whether a protein has a secretory leader as well as the cleavage point for that leader sequence are available. For instance, the method of McGeoch (*Virus Res.* 3:271-286 (1985)) uses the information from a short N-terminal charged region and a subsequent uncharged region of the complete (uncleaved) protein. The method of von Heinje (*Nucleic Acids Res.* 14:4683-4690 (1986)) uses the information from the residues surrounding the cleavage site, typically residues -13 to +2 where +1 indicates the amino terminus of the mature protein. The accuracy of predicting the cleavage points of known mammalian secretory proteins for each of these methods is in the range of 75-80% (von Heinje, *supra*). However, the two methods do not always produce the same predicted cleavage point(s) for a given protein.

In the present case, the deduced amino acid sequence of the complete TNFR polypeptides were analyzed by a computer program "PSORT", available from Dr. Kenta Nakai of the Institute for Chemical Research, Kyoto University

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(see K. Nakai and M. Kanehisa, *Genomics* 14:897-911 (1992)), which is an expert system for predicting the cellular location of a protein based on the amino acid sequence. As part of this computational prediction of localization, the methods of McGeoch and von Heinje are incorporated. The analysis of the TNFR amino acid sequences by this program provided the following results: TNFR-6 α & -6 β encode mature polypeptides having the amino acid sequences of residues 31-300 and 31-170 of SEQ ID NOS:2 and 4, respectively.

As indicated, nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA or RNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

Isolated nucleic acid molecules of the present invention include DNA molecules comprising an open reading frame (ORF) with an initiation codon at positions 25-27 73-75 of the nucleotide sequences shown in SEQ ID NOS:1 and 3, respectively.

Also included are DNA molecules comprising the coding sequence for the predicted mature TNFR polypeptides shown at positions 31-300 and 31-170 of SEQ ID NOS:2 and 4, respectively.

In addition, isolated nucleic acid molecules of the invention include DNA molecules which comprise a sequence substantially different from those described above but which, due to the degeneracy of the genetic code, still encode a TNFR protein. Of course, the genetic code and species-specific codon preferences are well known in the art. Thus, it would be routine for one skilled in the art to generate the degenerate variants described above, for instance, to

optimize codon expression for a particular host (e.g., change codons in the human mRNA to those preferred by a bacterial host such as *E. coli*).

In another aspect, the invention provides isolated nucleic acid molecules encoding a TNFR polypeptide having an amino acid sequence encoded by the cDNA clone contained in the plasmid deposited as ATCC Deposit No. 97810 or 97809. Preferably, this nucleic acid molecule will encode the mature polypeptide encoded by the above-described deposited cDNA clone.

The invention further provides an isolated nucleic acid molecule having the nucleotide sequence shown in Figure 1 or 2 (SEQ ID NO:1 or 3) or the nucleotide sequence of the TNFR cDNAs contained in the above-described deposited clones, or a nucleic acid molecule having a sequence complementary to one of the above sequences. Such isolated molecules, particularly DNA molecules, are useful as probes for gene mapping, by *in situ* hybridization with chromosomes, and for detecting expression of the TNFR genes in human tissue, for instance, by Northern blot analysis.

The present invention is further directed to nucleic acid molecules encoding portions of the nucleotide sequences described herein as well as to fragments of the isolated nucleic acid molecules described herein. In particular, the invention provides polynucleotides having a nucleotide sequence representing the portion of SEQ ID NO:1 or 3 which consist of positions 25-924 and 73-582 of SEQ ID NOS:1 and 3, respectively. Also contemplated are polynucleotides encoding TNFR polypeptides which lack an amino terminal methionine such polynucleotides having a nucleotide sequence representing the portion of SEQ ID NOS:1 and 3 which consist of positions 28-924 and 76-582, respectively. Polypeptides encoded by such polynucleotides are also provided, such polypeptides comprising an amino acid sequence at positions 2-300 and 2-170 of SEQ ID NOS:2 and 4, respectively.

In addition, the invention provides nucleic acid molecules having nucleotide sequences related to extensive portions of SEQ ID NOS:1 and 3 as follows: HELDI06R (SEQ ID NO:17) and HCEOW38R (SEQ ID NO:18) are related to both SEQ ID NOS:1 and 3. Preferred are polypeptide fragments of SEQ ID NOS:1 and 3 which are not SEQ ID NO:19 or 20 or subfragments of either SEQ ID NO:19 or 20. The sequences of HELDI06R and HCEOW38R are shown in Figure 6.

More generally, by a fragment of an isolated nucleic acid molecule having the nucleotide sequence of the deposited cDNA or the nucleotide sequence shown in Figures 1 or 2 (SEQ ID NOS:1 or 3) is intended fragments at least about 15 nt, and more preferably at least about 20 nt, still more

preferably at least about 30 nt, and even more preferably, at least about 40 nt in length which are useful as diagnostic probes and primers as discussed herein. Of course, larger fragments 50-300 nt in length are also useful according to the present invention as are fragments corresponding to most, if not all, of the nucleotide sequence of the deposited cDNAs or as shown in Figures 1 and 2 (SEQ ID NOS:1 and 3). Especially preferred are fragments comprising at least 500 nucleotides which are at least 95% identical to 500 contiguous nucleotides shown in SEQ ID NO:1. By a fragment at least 20 nt in length, for example, is intended fragments which include 20 or more contiguous bases from the nucleotide sequence of a deposited cDNA or the nucleotide sequence as shown in Figures 1 and 2 (SEQ ID NOS:1 and 3). Preferred nucleic acid fragments of the present invention include nucleic acid molecules encoding epitope-bearing portions of the TNFR polypeptides as identified in Figures 4 and 5 and described in more detail below.

In another aspect, the invention provides an isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a portion of the polynucleotide in a nucleic acid molecule of the invention described above, for instance, a cDNA clone contained in ATCC Deposit No. 97810 or 97809. By "stringent hybridization conditions" is intended overnight incubation at 42° C in a solution comprising: 50% formamide, 5x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65° C.

By a polynucleotide which hybridizes to a "portion" of a polynucleotide is intended a polynucleotide (either DNA or RNA) hybridizing to at least about 15 nucleotides (nt), and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably about 30-70 (e.g., 50) nt of the reference polynucleotide. These are useful as diagnostic probes and primers as discussed above and in more detail below.

By a portion of a polynucleotide of "at least 20 nt in length," for example, is intended 20 or more contiguous nucleotides from the nucleotide sequence of the reference polynucleotide (e.g., a deposited cDNA or a nucleotide sequence as shown in Figure 1 or 2 (SEQ ID NO:1 or 3)). Of course, a polynucleotide which hybridizes only to a poly A sequence (such as the 3' terminal poly(A) tract of a TNFR cDNA, or to a complementary stretch of T (or U) residues, would not be included in a polynucleotide of the invention used to hybridize to a portion of a nucleic acid of the invention, since such a polynucleotide would hybridize to any nucleic acid molecule containing a poly

(A) stretch or the complement thereof (e.g., practically any double-stranded cDNA clone).

As indicated, nucleic acid molecules of the present invention which encode a TNFR polypeptide may include, but are not limited to those encoding the amino acid sequence of the mature polypeptide, by itself; and the coding sequence for the mature polypeptide and additional sequences, such as those encoding the about 26-35 amino acid leader or secretory sequence, such as a pre-, or pro- or prepro- protein sequence; the coding sequence of the mature polypeptide, with or without the aforementioned additional coding sequences.

Also encoded by nucleic acids of the invention are the above protein sequences together with additional, non-coding sequences, including for example, but not limited to introns and non-coding 5' and 3' sequences, such as the transcribed, non-translated sequences that play a role in transcription, mRNA processing, including splicing and polyadenylation signals, for example - ribosome binding and stability of mRNA; an additional coding sequence which codes for additional amino acids, such as those which provide additional functionalities.

Thus, the sequence encoding the polypeptide may be fused to a marker sequence, such as a sequence encoding a peptide which facilitates purification of the fused polypeptide. In certain preferred embodiments of this aspect of the invention, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311), among others, many of which are commercially available. As described in Gentz *et al.*, *Proc. Natl. Acad. Sci. USA* 86:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. The "HA" tag is another peptide useful for purification which corresponds to an epitope derived from the influenza hemagglutinin protein, which has been described by Wilson *et al.*, *Cell* 37: 767 (1984). As discussed below, other such fusion proteins include a TNFR-5, -6 α or -6 β fused to Fc at the N- or C-terminus.

Variant and Mutant Polynucleotides

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of a TNFR polypeptide. Variants may occur naturally, such as a natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. *Genes II*, Lewin, B., ed., John Wiley & Sons, New York (1985).

Non-naturally occurring variants may be produced using art-known mutagenesis techniques.

Such variants include those produced by nucleotide substitutions, deletions or additions. The substitutions, deletions or additions may involve one or more nucleotides. The variants may be altered in coding regions, non-coding regions, or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the TNFR polypeptide or portions thereof. Also especially preferred in this regard are conservative substitutions.

Highly preferred are nucleic acid molecules encoding a mature protein having an amino acid sequence shown in SEQ ID NOS:2 and 4 or the mature TNFR polypeptide sequences encoded by the deposited cDNA clones.

Most highly preferred are nucleic acid molecules encoding the extracellular domain of a protein having the amino acid sequence shown in SEQ ID NO:2 or 4 or the extracellular domain of a TNFR amino acid sequence encoded by a deposited cDNA clone.

Further embodiments include an isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence at least 90% identical, and more preferably at least 95%, 96%, 97%, 98% or 99% identical to a polynucleotide selected from the group consisting of: (a) a nucleotide sequence encoding a TNFR polypeptide having the complete amino acid sequence in SEQ ID NO:2 or 4, or as encoded by a cDNA clone contained in ATCC Deposit No. 97810 or 97809; (b) a nucleotide sequence encoding a mature TNFR polypeptide having an amino acid sequence at positions 31-300 or 31-170 in SEQ ID NO:2 or 4, respectively, or as encoded by a cDNA clone contained in ATCC Deposit No. 97810 or 97809; (c) a nucleotide sequence encoding a soluble extracellular domain of a TNFR polypeptide having the amino acid sequence at positions 31-283 and 31-166 of SEQ ID NOS:2 and 4, respectively; and (d) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b) or (c) above.

Further embodiments of the invention include isolated nucleic acid molecules that comprise a polynucleotide having a nucleotide sequence at least 90% identical, and more preferably at least 95%, 96%, 97%, 98% or 99% identical, to any of the nucleotide sequences in (a), (b), (c), or (d), above, or a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide in (a), (b), (c), or (d), above. This polynucleotide which hybridizes does not hybridize under stringent hybridization conditions to a

polynucleotide having a nucleotide sequence consisting of only A residues or of only T residues. An additional nucleic acid embodiment of the invention relates to an isolated nucleic acid molecule comprising a polynucleotide which encodes the amino acid sequence of an epitope-bearing portion of a TNFR polypeptide having an amino acid sequence in (a), (b), (c) or (d), above.

The present invention also relates to recombinant vectors, which include the isolated nucleic acid molecules of the present invention, and to host cells containing the recombinant vectors, as well as to methods of making such vectors and host cells and for using them for production of TNFR polypeptides or peptides by recombinant techniques.

By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence encoding a TNFR polypeptide is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding the TNFR polypeptide. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular nucleic acid molecule is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, a nucleotide sequence shown in Figure 1 or 2, or to the nucleotides sequence of a deposited cDNA clone can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). Bestfit uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics* 2:482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence

and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

The present application is directed to nucleic acid molecules at least 90%, 95%, 96%, 97%, 98% or 99% identical to a nucleic acid sequence shown in Figure 1 or 2 (SEQ ID NO:1 and 3) or to the nucleic acid sequence of a deposited cDNA, irrespective of whether they encode a polypeptide having TNFR activity. This is because even where a particular nucleic acid molecule does not encode a polypeptide having TNFR activity, one of skill in the art would still know how to use the nucleic acid molecule, for instance, as a hybridization probe or a polymerase chain reaction (PCR) primer. Uses of the nucleic acid molecules of the present invention that do not encode a polypeptide having TNFR activity include, inter alia, (1) isolating a TNFR gene or allelic variants thereof in a cDNA library; (2) *in situ* hybridization (e.g., "FISH") to metaphase chromosomal spreads to provide precise chromosomal location of the TNFR gene, as described in Verma et al., Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York (1988); and Northern Blot analysis for detecting TNFR mRNA expression in specific tissues.

Preferred, however, are nucleic acid molecules having sequences at least 90%, 95%, 96%, 97%, 98% or 99% identical to a nucleic acid sequence shown in Figure 1 or 2 (SEQ ID NOS:1 and 3) or to the nucleic acid sequence of a deposited cDNA which do, in fact, encode polypeptides having TNFR protein activity. By "a polypeptide having TNFR activity" is intended polypeptides exhibiting activity similar, but not necessarily identical, to an activity of a mature or extracellular forms of a TNFR-6 α or -6 β protein of the invention, as measured in a particular biological assay. The TNF family ligands induce various cellular responses by binding to TNF-family receptors, including the TNFR-6 α and -6 β of the present invention. Cells which express the TNFR proteins are believed to have a potent cellular response to TNFR-I receptor ligands including B lymphocytes (CD19+), both CD4 and CD8+ T lymphocytes, monocytes and endothelial cells. By a "cellular response to a TNF-family ligand" is intended any genotypic, phenotypic, and/or morphological change to a cell, cell line, tissue, tissue culture or patient that is induced by a TNF-family ligand. As indicated, such cellular responses include not only normal physiological responses to TNF-family ligands, but also diseases associated with increased cell proliferation or the inhibition of increased cell proliferation, such as by the inhibition of apoptosis.

Screening assays for the forgoing are known in the art. One such screening assay involves the use of cells which express the receptor (for

example, transfected CHO cells) in a system which measures extracellular pH changes caused by receptor activation, for example, as described in Science 246:181-296 (October 1989). For example, a TNF-family ligand may be contacted with a cell which expresses the mature form of the receptor polypeptide of the present invention and a second messenger response, e.g., signal transduction or pH changes, may be measured to determine whether the TNFR polypeptide is active.

Of course, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of the nucleic acid molecules having a sequence at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the nucleic acid sequence of a deposited cDNA or the nucleic acid sequence shown in Figure 1 or 2 (SEQ ID NO:1 and 3) will encode a polypeptide "having TNFR protein activity." In fact, since degenerate variants of these nucleotide sequences all encode the same polypeptide, this will be clear to the skilled artisan even without performing the above described comparison assay. It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a polypeptide having TNFR protein activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly effect protein function (e.g., replacing one aliphatic amino acid with a second aliphatic amino acid), as further described below.

Vectors and Host Cells

The present invention also relates to vectors which include the isolated DNA molecules of the present invention, host cells which are genetically engineered with the recombinant vectors, and the production of TNFR polypeptides or fragments thereof by recombinant techniques. The vector may be, for example, a phage, plasmid, viral or retroviral vector. Retroviral vectors may be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing host cells.

The polynucleotides may be joined to a vector containing a selectable marker for propagation in a host. Generally, a plasmid vector is introduced in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. If the vector is a virus, it may be packaged in vitro using an appropriate packaging cell line and then transduced into host cells.

The DNA insert should be operatively linked to an appropriate promoter, such as the phage lambda PL promoter, the *E. coli lac*, *trp*, *phoA* and *tac* promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name a few. Other suitable promoters will be known to the skilled artisan.

The expression constructs will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. The coding portion of the transcripts expressed by the constructs will preferably include a translation initiating codon at the beginning and a
5 termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

As indicated, the expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture and tetracycline, kanamycin or ampicillin resistance genes for culturing in *E. coli* and other bacteria.

5 Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, 293 and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions
10 for the above-described host cells are known in the art. Among vectors preferred for use in bacteria include pQE70, pQE60 and pQE-9, available from QIAGEN, Inc., *supra*; Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among
15 preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic
20 lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis *et al.*, *Basic Methods In Molecular Biology* (1986). The polypeptide may be expressed in a modified form, such as a fusion protein, and may include not only secretion signals, but also additional heterologous
25 functional regions. For instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification, or during subsequent handling and storage. Also, peptide moieties may be added to the polypeptide to facilitate purification. Such regions may be removed prior to
30 final preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability and to facilitate purification, among others, are familiar and routine techniques in the art. A preferred fusion protein comprises a heterologous region from immunoglobulin that is useful to stabilize and purify proteins. For example,
35 EP-A-O 464 533 (Canadian counterpart 2045869) discloses fusion proteins comprising various portions of constant region of immunoglobulin molecules together with another human protein or part thereof. In many cases, the Fc part in a fusion protein is thoroughly advantageous for use in therapy and diagnosis and thus results, for example, in improved pharmacokinetic properties (EP-A

0232 262). On the other hand, for some uses it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected and purified in the advantageous manner described. This is the case when Fc portion proves to be a hindrance to use in therapy and diagnosis, for example when the fusion protein is to be used as antigen for immunizations. In drug discovery, for example, human proteins, such as hIL-5, have been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists of hIL-5. See, D. Bennett *et al.*, *J. Molecular Recognition* 8:52-58 (1995) and K. Johanson *et al.*, *J. Biol. Chem.* 270:9459-9471 (1995).

The TNFR proteins can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification. Polypeptides of the present invention include: products purified from natural sources, including bodily fluids, tissues and cells, whether directly isolated or cultured; products of chemical synthetic procedures; and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

Polypeptides and Fragments

The invention further provides isolated TNFR polypeptides having the amino acid sequences encoded by the deposited cDNAs, or the amino acid sequences in SEQ ID NOS:2 and 4, or a peptide or polypeptide comprising a portion of the above polypeptides.

Variant and Mutant Polypeptides

To improve or alter the characteristics of a TNFR polypeptide, protein engineering may be employed. Recombinant DNA technology known to those skilled in the art can be used to create novel mutant proteins or "muteins" including single or multiple amino acid substitutions, deletions, additions or fusion proteins. Such modified polypeptides can show, e.g., enhanced activity

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or increased stability. In addition, they may be purified in higher yields and show better solubility than the corresponding natural polypeptide, at least under certain purification and storage conditions.

N-Terminal and C-Terminal Deletion Mutants

5 For instance, for many proteins, including the extracellular domain of a membrane associated protein or the mature form(s) of a secreted protein, it is known in the art that one or more amino acids may be deleted from the N-terminus or C-terminus without substantial loss of biological function. For instance, Ron et al., *J. Biol. Chem.*, 268:2984-2988 (1993) reported modified
10 KGF proteins that had heparin binding activity even if 3, 8, or 27 amino-terminal amino acid residues were missing. In the present case, since the proteins of the invention are members of the TNFR polypeptide family, deletions of N-terminal amino acids up to the Cysteine at position 49 of SEQ ID NOS:2 and 4 (TNFR-6 α and -6 β) may retain some biological activity such as
15 regulation of proliferation and apoptosis of lymphoid cells. Polypeptides having further N-terminal deletions including the C49 residue in SEQ ID NOS:2 and 4, would not be expected to retain such biological activities because it is known that these residues in a TNFR-related polypeptide are required for forming a disulfide bridge to provide structural stability which is needed for
20 receptor binding and signal transduction.

However, even if deletion of one or more amino acids from the N-terminus of a protein results in modification or loss of one or more biological functions of the protein, other biological activities may still be retained. Thus, the ability of the shortened protein to induce and/or bind to antibodies which
25 recognize the complete or extracellular domain of the TNFR protein generally will be retained when less than the majority of the residues of the complete protein or extracellular domain are removed from the N-terminus. Whether a particular polypeptide lacking N-terminal residues of a complete protein retains such immunologic activities can readily be determined by routine methods
30 described herein and otherwise known in the art.

Accordingly, the present invention further provides polypeptides having one or more residues deleted from the amino terminus of the amino acid sequence of the TNFR shown in SEQ ID NOS:2 and 4, up to the cysteine residue at position number 49, and polynucleotides encoding such polypeptides.
35 In particular, the present invention provides TNFR-5 polypeptides comprising the amino acid sequence of residues m-300 and n-170 of SEQ ID NOS:2 and 4, respectively where m and n are integers in the range of 1-49 where 49 is the

position of the first cysteine residue from the N-terminus of the complete TNFR-6 α and -6 β polypeptides (shown in SEQ ID NOS:2 and 4, respectively) believed to be required for activity of the TNFR-6 α and -6 β proteins.

More in particular, the invention provides polynucleotides encoding polypeptides having the amino acid sequence of residues: 1-300, 2-300, 3-300, 4-300, 5-300, 6-300, 7-300, 8-300, 9-300, 10-300, 11-300, 12-300, 13-300, 14-300, 15-300, 16-300, 17-300, 18-300, 19-300, 20-300, 21-300, 22-300, 23-300, 24-300, 25-300, 26-300, 27-300, 28-300, 29-300, 30-300, 31-300, 32-300, 33-300, 34-300, 35-300, 36-300, 37-300, 38-300, 39-300, 40-300, 41-300, 42-300, 43-300, 44-300, 45-300, 46-300, 47-300, 48-300, and 49-300 of SEQ ID NO:2; and 1-170, 2-170, 3-170, 4-170, 5-170, 6-170, 7-170, 8-170, 9-170, 10-170, 11-170, 12-170, 13-170, 14-170, 15-170, 16-170, 17-170, 18-170, 19-170, 20-170, 21-170, 22-170, 23-170, 24-170, 25-170, 26-170, 27-170, 28-170, 29-170, 30-170, 31-170, 32-170, 33-170, 34-170, 35-170, 36-170, 37-170, 38-170, 39-170, 40-170, 41-170, 42-170, 43-170, 44-170, 45-170, 46-170, 47-170, 48-170, and 49-170 of SEQ ID NO:4.

Polynucleotides encoding these polypeptides also are provided.

Similarly, many examples of biologically functional C-terminal deletion mutants are known. For instance, interferon gamma shows up to ten times higher activities by deleting 8-10 amino acid residues from the carboxy terminus of the protein (Döbeli et al., *J. Biotechnology* 7:199-216 (1988)). In the present case, since the protein of the invention is a member of the TNFR polypeptide family, deletions of C-terminal amino acids up to the cysteine at position 193 and 132 of SEQ ID NOS:2 and 4, respectively, may retain some biological activity such as regulation of proliferation and apoptosis of lymphoid cells. Polypeptides having further C-terminal deletions including the cysteines at positions 193 and 132 of SEQ ID NOS:2 and 4, respectively, would not be expected to retain such biological activities because it is known that these residues in TNF receptor-related polypeptides are required for forming disulfide bridges to provide structural stability which is needed for receptor binding.

However, even if deletion of one or more amino acids from the C-terminus of a protein results in modification or loss of one or more biological functions of the protein, other biological activities may still be retained. Thus, the ability of the shortened protein to induce and/or bind to antibodies which recognize the complete or mature form of the protein generally will be retained when less than the majority of the residues of the complete or mature form protein are removed from the C-terminus. Whether a particular polypeptide lacking C-terminal residues of a complete protein retains such immunologic

activities can readily be determined by routine methods described herein and otherwise known in the art.

Accordingly, the present invention further provides polypeptides having one or more residues from the carboxy terminus of the amino acid sequence of TNFR-6 α and -6 β shown in SEQ ID NOS:2 and 4 up to the cysteine at position 193 and 132 of SEQ ID NOS:2 and 4, respectively, and polynucleotides encoding such polypeptides. In particular, the present invention provides polypeptides having the amino acid sequence of residues 1-y and 1-z of the amino acid sequence in SEQ ID NOS:2 and 4, respectively, where y is any integer in the range of 193-300 and z is any integer in the range of 132-170. Polynucleotides encoding these polypeptides also are provided.

The invention also provides polypeptides having one or more amino acids deleted from both the amino and the carboxyl termini, which may be described generally as having residues m-y of SEQ ID NO:2 and n-z of SEQ ID NO:4, where m, n, y and z are integers as described above.

Also included are a nucleotide sequence encoding a polypeptide consisting of a portion of a complete TNFR amino acid sequence encoded by a cDNA clone contained in ATCC Deposit No. 97810, or 97809, where this portion excludes from 1 to about 49 amino acids from the amino terminus of the complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97810 and 97809, respectively, or from 1 to about 107 or 58 amino acids from the carboxy terminus of the complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97810 and 97809, respectively, or any combination of the above amino terminal and carboxy terminal deletions, of the complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97810 or 97809. Polynucleotides encoding all of the above deletion mutant polypeptide forms also are provided.

Other Mutants

In addition to terminal deletion forms of the protein discussed above, it also will be recognized by one of ordinary skill in the art that some amino acid sequences of the TNFR polypeptides can be varied without significant effect on the structure or function of the proteins. If such differences in sequence are contemplated, it should be remembered that there will be critical areas on the protein which determine activity.

Thus, the invention further includes variations of the TNFR polypeptides which show substantial TNFR polypeptide activity or which include regions of TNFR protein such as the protein portions discussed below. Such mutants include

deletions, insertions, inversions, repeats, and type substitutions selected according to general rules known in the art so as have little effect on activity. For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, J. U. et al., "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science* 247:1306-1310 (1990), wherein the authors indicate that there are two main approaches for studying the tolerance of an amino acid sequence to change. The first method relies on the process of evolution, in which mutations are either accepted or rejected by natural selection. The second approach uses genetic engineering to introduce amino acid changes at specific positions of a cloned gene and selections or screens to identify sequences that maintain functionality. As the authors state, these studies have revealed that proteins are surprisingly tolerant of amino acid substitutions. The authors further indicate which amino acid changes are likely to be permissive at a certain position of the protein. For example, most buried amino acid residues require nonpolar side chains, whereas few features of surface side chains are generally conserved. Other such phenotypically silent substitutions are described in Bowie, J. U. et al., *supra*, and the references cited therein. Typically seen as conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile; interchange of the hydroxyl residues Ser and Thr, exchange of the acidic residues Asp and Glu, substitution between the amide residues Asn and Gln, exchange of the basic residues Lys and Arg and replacements among the aromatic residues Phe, Tyr. Thus, the fragment, derivative or analog of the polypeptide of SEQ ID NO:2, 4 or 6, or that encoded by a deposited cDNA, may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature or soluble extracellular polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the above form of the polypeptide, such as an IgG Fc fusion region peptide or leader or secretory sequence or a sequence which is employed for purification of the above form of the polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

Thus, the TNFR of the present invention may include one or more amino acid substitutions, deletions or additions, either from natural mutations or

human manipulation. As indicated, changes are preferably of a minor nature, such as conservative amino acid substitutions that do not significantly affect the folding or activity of the protein (see Table 1).

TABLE 1. Conservative Amino Acid Substitutions.

Aromatic	Phenylalanine Tryptophan Tyrosine
Hydrophobic	Leucine Isoleucine Valine
Polar	Glutamine Asparagine
Basic	Arginine Lysine Histidine
Acidic	Aspartic Acid Glutamic Acid
Small	Alanine Serine Threonine Methionine Glycine

Amino acids in the TNFR proteins of the present invention that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science* 244:1081-1085 (1989)). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for biological activity such as receptor binding or *in vitro* or *in vitro* proliferative activity.

Of special interest are substitutions of charged amino acids with other charged or neutral amino acids which may produce proteins with highly desirable improved characteristics, such as less aggregation. Aggregation may not only reduce activity but also be problematic when preparing pharmaceutical formulations, because aggregates can be immunogenic (Pinckard *et al.*, *Clin. Exp. Immunol.* 2:331-340 (1967); Robbins *et al.*, *Diabetes* 36: 838-845 (1987); Cleland *et al.*, *Crit. Rev. Therapeutic Drug Carrier Systems* 10:307-377 (1993).

Replacement of amino acids can also change the selectivity of the binding of a ligand to cell surface receptors. For example, Ostade *et al.*, *Nature* 361:266-268 (1993) describes certain mutations resulting in selective binding of TNF- α to only one of the two known types of TNF receptors. Sites that are critical for ligand-receptor binding can also be determined by structural analysis such as crystallization, nuclear magnetic resonance or photoaffinity labeling

(Smith *et al.*, *J. Mol. Biol.* 224:899-904 (1992) and de Vos *et al. Science* 255:306-312 (1992)).

Since TNFR-6 α and -6 β are members of the TNF receptor-related protein family, to modulate rather than completely eliminate biological activities of TNFR preferably mutations are made in sequences encoding amino acids in the TNFR conserved extracellular domain, more preferably in residues within this region which are not conserved among members of the TNF receptor family. Also forming part of the present invention are isolated polynucleotides comprising nucleic acid sequences which encode the above TNFR mutants.

The polypeptides of the present invention are preferably provided in an isolated form, and preferably are substantially purified. A recombinantly produced version of the TNFR polypeptides can be substantially purified by the one-step method described in Smith and Johnson, *Gene* 67:31-40 (1988). Polypeptides of the invention also can be purified from natural or recombinant sources using anti-TNFR-6 α and -6 β antibodies of the invention in methods which are well known in the art of protein purification.

The invention further provides isolated TNFR polypeptides comprising an amino acid sequence selected from the group consisting of: (a) the amino acid sequence of a full-length TNFR polypeptide having the complete amino acid sequence shown in SEQ ID NO:2 or 4 or as encoded by a cDNA clone contained in ATCC Deposit No. 97810 or 97809; (b) the amino acid sequence of a mature TNFR polypeptide having the amino acid sequence at positions 31-300 in SEQ ID NO:2 or 31-170 in SEQ ID NO:4, or as encoded by a cDNA clone contained in ATCC Deposit No. 97810 or 97809; or (c) the amino acid sequence of a soluble extracellular domain of a TNFR polypeptide having the amino acid sequence at positions 31 to 283 in SEQ ID NO:2 or 31 to 166 in SEQ ID NO:4, or as encoded by the cDNA clone contained in ATCC Deposit No. 97810 or 97809.

Further polypeptides of the present invention include polypeptides which have at least 90% similarity, more preferably at least 95% similarity, and still more preferably at least 96%, 97%, 98% or 99% similarity to those described above. The polypeptides of the invention also comprise those which are at least 80% identical, more preferably at least 90% or 95% identical, still more preferably at least 96%, 97%, 98% or 99% identical to the polypeptide encoded by the deposited cDNA or to the polypeptide of SEQ ID NO:2 or 4, and also include portions of such polypeptides with at least 30 amino acids and more preferably at least 50 amino acids.

By "% similarity" for two polypeptides is intended a similarity score produced by comparing the amino acid sequences of the two polypeptides using the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711) and the default settings for determining similarity. Bestfit uses the local homology algorithm of Smith and Waterman (Advances in Applied Mathematics 2:482-489, 1981) to find the best segment of similarity between two sequences.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a reference amino acid sequence of a TNFR polypeptide is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference amino acid of the TNFR polypeptide. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular polypeptide is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in SEQ ID NO:2 or 4, or to an amino acid sequence encoded by a deposited cDNA clone can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

The polypeptide of the present invention could be used as a molecular weight marker on SDS-PAGE gels or on molecular sieve gel filtration columns using methods well known to those of skill in the art.

As described in detail below, the polypeptides of the present invention can also be used to raise polyclonal and monoclonal antibodies, which are useful in assays for detecting TNFR protein expression as described below or as agonists and antagonists capable of enhancing or inhibiting TNFR protein function. Further, such polypeptides can be used in the yeast two-hybrid system to "capture" TNFR protein binding proteins which are also candidate agonists and antagonists according to the present invention. The yeast two hybrid system is described in Fields and Song, *Nature* 340:245-246 (1989).

Epitope-Bearing Portions

In another aspect, the invention provides a peptide or polypeptide comprising an epitope-bearing portion of a polypeptide of the invention. The epitope of this polypeptide portion is an immunogenic or antigenic epitope of a polypeptide of the invention. An "immunogenic epitope" is defined as a part of a protein that elicits an antibody response when the whole protein is the immunogen. On the other hand, a region of a protein molecule to which an antibody can bind is defined as an "antigenic epitope." The number of immunogenic epitopes of a protein generally is less than the number of antigenic epitopes. See, for instance, Geysen *et al.*, *Proc. Natl. Acad. Sci. USA* 81:3998- 4002 (1983).

As to the selection of peptides or polypeptides bearing an antigenic epitope (i.e., that contain a region of a protein molecule to which an antibody can bind), it is well known in that art that relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See, for instance, Sutcliffe, J. G., Shinnick, T. M., Green, N. and Learner, R. A. (1983) "Antibodies that react with predetermined sites on proteins," *Science*, 219:660-666. Peptides capable of eliciting protein-reactive sera are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined neither to immunodominant regions of intact proteins (i.e., immunogenic epitopes) nor to the amino or carboxyl terminals. Antigenic epitope-bearing peptides and polypeptides of the invention are therefore useful to raise antibodies, including monoclonal antibodies, that bind specifically to a polypeptide of the invention. See, for instance, Wilson *et al.*, *Cell* 37:767-778 (1984) at 777.

Antigenic epitope-bearing peptides and polypeptides of the invention preferably contain a sequence of at least seven, more preferably at least nine and most preferably between about 15 to about 30 amino acids contained within the amino acid sequence of a polypeptide of the invention. Non-limiting

examples of antigenic polypeptides or peptides that can be used to generate TNFR-specific antibodies include: a polypeptide comprising amino acid residues from about Ala-31 to about Thr-46, from about Phe-57 to about Thr-117, from about Cys-132 to about Thr-175, from about Gly-185 to about Thr-194, from about Val-205 to about Asp-217, from about Pro-239 to about Leu-264, and from about Ala-283 to about Pro-298 in SEQ ID NO:2; and from about Ala-31 to about Thr-46, from about Phe-57 to about Gln-80, from about Glu-86 to about His-106, from about Thr-108 to about Phe-119, from about His-129 to about Val-138, and from about Gly-142 to about Pro-166 in SEQ ID NO:4. These polypeptide fragments have been determined to bear antigenic epitopes of the TNFR-6 α and -6 β polypeptides respectively, by the analysis of the Jameson-Wolf antigenic index, as shown in Figures 4 and 5, above.

The epitope-bearing peptides and polypeptides of the invention may be produced by any conventional means. See, e.g., Houghten, R. A. (1985) "General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids." *Proc. Natl. Acad. Sci. USA* 82:5131-5135; this "Simultaneous Multiple Peptide Synthesis (SMPS)" process is further described in U.S. Patent No. 4,631,211 to Houghten *et al.* (1986).

Epitope-bearing peptides and polypeptides of the invention are used to induce antibodies according to methods well known in the art. See, for instance, Sutcliffe *et al.*, *supra*; Wilson *et al.*, *supra*; Chow, M. *et al.*, *Proc. Natl. Acad. Sci. USA* 82:910-914; and Bittle, F. J. *et al.*, *J. Gen. Virol.* 66:2347-2354 (1985). Immunogenic epitope-bearing peptides of the invention, i.e., those parts of a protein that elicit an antibody response when the whole protein is the immunogen, are identified according to methods known in the art. See, for instance, Geysen *et al.*, *supra*. Further still, U.S. Patent No. 5,194,392 to Geysen (1990) describes a general method of detecting or determining the sequence of monomers (amino acids or other compounds) which is a topological equivalent of the epitope (i.e., a "mimotope") which is complementary to a particular paratope (antigen binding site) of an antibody of interest. More generally, U.S. Patent No. 4,433,092 to Geysen (1989) describes a method of detecting or determining a sequence of monomers which is a topographical equivalent of a ligand which is complementary to the ligand binding site of a particular receptor of interest. Similarly, U.S. Patent No. 5,480,971 to Houghten, R. A. *et al.* (1996) on Peralkylated Oligopeptide Mixtures discloses linear C1-C7-alkyl peralkylated oligopeptides and sets and libraries of such peptides, as well as methods for using such oligopeptide sets

and libraries for determining the sequence of a peralkylated oligopeptide that preferentially binds to an acceptor molecule of interest. Thus, non-peptide analogs of the epitope-bearing peptides of the invention also can be made routinely by these methods.

Fusion Proteins

As one of skill in the art will appreciate, TNFR polypeptides of the present invention and the epitope-bearing fragments thereof described above can be combined with parts of the constant domain of immunoglobulins (IgG), resulting in chimeric polypeptides. These fusion proteins facilitate purification and show an increased half-life in vivo. This has been shown, e.g., for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins (EP A 394,827; Traunecker et al., Nature 331:84-86 (1988)). Fusion proteins that have a disulfide-linked dimeric structure due to the IgG part can also be more efficient in binding and neutralizing other molecules than the monomeric TNFR protein or protein fragment alone (Fountoulakis et al., J. Biochem. 270:3958-3964 (1995)).

Antibodies

TNFR-protein specific antibodies for use in the present invention can be raised against the intact TNFR-6 α and -6 β proteins or an antigenic polypeptide fragment thereof, which may be presented together with a carrier protein, such as an albumin, to an animal system (such as rabbit or mouse) or, if it is long enough (at least about 25 amino acids), without a carrier.

As used herein, the term "antibody" (Ab) or "monoclonal antibody" (Mab) is meant to include intact molecules as well as antibody fragments (such as, for example, Fab and F(ab')₂ fragments) which are capable of specifically binding to a TNFR protein. Fab and F(ab')₂ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl et al., J. Nucl. Med. 24:316-325 (1983)). Thus, these fragments are preferred.

The antibodies of the present invention may be prepared by any of a variety of methods. For example, cells expressing the TNFR protein or an antigenic fragment thereof can be administered to an animal in order to induce the production of sera containing polyclonal antibodies. In a preferred method, a preparation of TNFR protein is prepared and purified to render it substantially

free of natural contaminants. Such a preparation is then introduced into an animal in order to produce polyclonal antisera of greater specific activity.

In the most preferred method, the antibodies of the present invention are monoclonal antibodies. Such monoclonal antibodies can be prepared using hybridoma technology (Köhler *et al.*, *Nature* 256:495 (1975); Köhler *et al.*, *Eur. J. Immunol.* 6:511 (1976); Köhler *et al.*, *Eur. J. Immunol.* 6:292 (1976); Hammerling *et al.*, in: *Monoclonal Antibodies and T-Cell Hybridomas*, Elsevier, N.Y., (1981) pp. 563-681). In general, such procedures involve immunizing an animal (preferably a mouse) with a TNFR protein antigen or, more preferably, with a TNFR protein-expressing cell. Suitable cells can be recognized by their capacity to bind anti-TNFR-6 α or -6 β protein antibody.

Such cells may be cultured in any suitable tissue culture medium; however, it is preferable to culture cells in Earle's modified Eagle's medium supplemented with 10% fetal bovine serum (inactivated at about 56° C), and supplemented with about 10 g/l of nonessential amino acids, about 1,000 U/ml of penicillin, and about 100 μ g/ml of streptomycin. The splenocytes of such mice are extracted and fused with a suitable myeloma cell line. Any suitable myeloma cell line may be employed in accordance with the present invention; however, it is preferable to employ the parent myeloma cell line (SP2O), available from the American Type Culture Collection, Rockville, Maryland. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium, and then cloned by limiting dilution as described by Wands *et al.* (*Gastroenterology* 80:225-232 (1981)). The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding the desired TNFR antigen.

Alternatively, additional antibodies capable of binding to the TNFR antigen may be produced in a two-step procedure through the use of anti-idiotypic antibodies. Such a method makes use of the fact that antibodies are themselves antigens, and that, therefore, it is possible to obtain an antibody which binds to a second antibody. In accordance with this method, TNFR-protein specific antibodies are used to immunize an animal, preferably a mouse. The splenocytes of such an animal are then used to produce hybridoma cells, and the hybridoma cells are screened to identify clones which produce an antibody whose ability to bind to the TNFR protein-specific antibody can be blocked by the TNFR protein antigen. Such antibodies comprise anti-idiotypic antibodies to the TNFR protein-specific antibody and can be used to immunize an animal to induce formation of further TNFR protein-specific antibodies.

It will be appreciated that Fab and F(ab')₂ and other fragments of the antibodies of the present invention may be used according to the methods disclosed herein. Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')₂ fragments). Alternatively, TNFR protein-binding fragments can be produced through the application of recombinant DNA technology or through synthetic chemistry.

For *in vivo* use of anti-TNFR in humans, it may be preferable to use "humanized" chimeric monoclonal antibodies. Such antibodies can be produced using genetic constructs derived from hybridoma cells producing the monoclonal antibodies described above. Methods for producing chimeric antibodies are known in the art. See, for review, Morrison, *Science* 229:1202 (1985); Oi et al., *BioTechniques* 4:214 (1986); Cabilly et al., U.S. Patent No. 4,816,567; Taniguchi et al., EP 171496; Morrison et al., EP 173494; Neuberger et al., WO 8601533; Robinson et al., WO 8702671; Boulianne et al., *Nature* 312:643 (1984); Neuberger et al., *Nature* 314:268 (1985).

Immune System-Related Disorders

Diagnosis

The present inventors have discovered that TNFR-6 α and -6 β are expressed in hematopoietic and transformed tissues. For a number of immune system-related disorders, substantially altered (increased or decreased) levels of TNFR gene expression can be detected in immune system tissue or other cells or bodily fluids (e.g., sera and plasma) taken from an individual having such a disorder, relative to a "standard" TNFR gene expression level, that is, the TNFR expression level in immune system tissues or bodily fluids from an individual not having the immune system disorder. Thus, the invention provides a diagnostic method useful during diagnosis of an immune system disorder, which involves measuring the expression level of the gene encoding the TNFR protein in immune system tissue or other cells or body fluid from an individual and comparing the measured gene expression level with a standard TNFR gene expression level, whereby an increase or decrease in the gene expression level compared to the standard is indicative of an immune system disorder.

In particular, it is believed that certain tissues in mammals with cancer express significantly reduced levels of the TNFR protein and mRNA encoding the TNFR when compared to a corresponding "standard" level. Further, it is believed that reduced levels of the TNFR protein can be detected in certain body

fluids (e.g., sera and plasma) from mammals with such a cancer when compared to sera from mammals of the same species not having the cancer.

Thus, the invention provides a diagnostic method useful during diagnosis of an immune system disorder, including cancers which involves measuring the expression level of the gene encoding the TNFR protein in immune system tissue or other cells or body fluid from an individual and comparing the measured gene expression level with a standard TNFR gene expression level, whereby an increase or decrease in the gene expression level compared to the standard is indicative of an immune system disorder.

Where a diagnosis of a disorder in the immune system including diagnosis of a tumor has already been made according to conventional methods, the present invention is useful as a prognostic indicator, whereby patients exhibiting depressed gene expression will experience a worse clinical outcome relative to patients expressing the gene at a level nearer the standard level.

By "assaying the expression level of the gene encoding a TNFR protein" is intended qualitatively or quantitatively measuring or estimating the level of the TNFR-6 α and/or -6 β protein or the level of the mRNA encoding the TNFR-6 α and/or -6 β protein in a first biological sample either directly (e.g., by determining or estimating absolute protein level or mRNA level) or relatively (e.g., by comparing to the TNFR protein level or mRNA level in a second biological sample). Preferably, the TNFR protein level or mRNA level in the first biological sample is measured or estimated and compared to a standard TNFR protein level or mRNA level, the standard being taken from a second biological sample obtained from an individual not having the disorder or being determined by averaging levels from a population of individuals not having a disorder of the immune system. As will be appreciated in the art, once standard TNFR protein levels or mRNA levels are known, they can be used repeatedly as a standard for comparison.

By "biological sample" is intended any biological sample obtained from an individual, body fluid, cell line, tissue culture, or other source which contains TNFR protein or mRNA. As indicated, biological samples include body fluids (such as sera, plasma, urine, synovial fluid and spinal fluid) which contain free extracellular domain(s) (or soluble form(s)) of a TNFR protein, immune system tissue, and other tissue sources found to express complete or extracellular domain of a TNFR. Methods for obtaining tissue biopsies and body fluids from mammals are well known in the art. Where the biological sample is to include mRNA, a tissue biopsy is the preferred source.

The invention also contemplates the use of a gene of the present invention for diagnosing mutations in a TNFR gene. For example, if a mutation is present in one of the genes of the present invention, conditions would result from a lack of production of the receptor polypeptides of the present invention. Further, mutations which enhance receptor polypeptide activity would lead to diseases associated with an over expression of the receptor polypeptide, e.g., endotoxic shock. Mutations in the genes can be detected by comparing the sequence of the defective gene with that of a normal one. Subsequently one can verify that a mutant gene is associated with a disease condition or the susceptibility to a disease condition. That is, a mutant gene which leads to the underexpression of the receptor polypeptides of the present invention would be associated with an inability of TNF to inhibit tumor growth.

Other immune system disorders which may be diagnosed by the foregoing assays include hypersensitivity, allergy, infectious disease, graft-host disease, immunodeficiency, autoimmune diseases and the like.

Individuals carrying mutations in the genes of the present invention may be detected at the DNA level by a variety of techniques. Nucleic acids used for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva and tissue biopsy among other tissues. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR (Saiki et al., Nature, 324:163-166 (1986)) prior to analysis. RNA or cDNA may also be used for the same purpose. As an example, PCR primers complementary to the nucleic acid of the instant invention can be used to identify and analyze mutations in the human genes of the present invention. For example, deletions and insertions can be detected by a change in the size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled RNA or alternatively, radiolabeled antisense DNA sequences of the present invention. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures. Such a diagnostic would be particularly useful for prenatal or even neonatal testing.

Sequence differences between the reference gene and "mutants" may be revealed by the direct DNA sequencing method. In addition, cloned DNA segments may be used as probes to detect specific DNA segments. The sensitivity of this method is greatly enhanced when combined with PCR. For example, a sequencing primary used with double stranded PCR product or a single stranded template molecule generated by a modified PCR product. The sequence determination is performed by conventional procedures with

radiolabeled nucleotides or by automatic sequencing procedures with fluorescent tags.

Sequence changes at the specific locations may be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (for example, Cotton et al., PNAS, 85:4397-4401 (1985)).

Assaying TNFR protein levels in a biological sample can occur using antibody-based techniques. For example, TNFR protein expression in tissues can be studied with classical immunohistological methods (Jalkanen, M., et al., *J. Cell. Biol.* 101:976-985 (1985); Jalkanen, M., et al., *J. Cell. Biol.* 105:3087-3096 (1987)). Other antibody-based methods useful for detecting TNFR gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). Suitable antibody assay labels are known in the art and include enzyme labels, such as, glucose oxidase, and radioisotopes, such as iodine (^{125}I , ^{121}I), carbon (^{14}C), sulfur (^{35}S), tritium (^3H), indium (^{112}In), and technetium ($^{99\text{m}}\text{Tc}$), and fluorescent labels, such as fluorescein and rhodamine, and biotin.

In addition to assaying TNFR protein levels in a biological sample obtained from an individual, TNFR proteins can also be detected *in vivo* by imaging. Antibody labels or markers for *in vivo* imaging of TNFR proteins include those detectable by X-radiography, NMR or ESR. For X-radiography, suitable labels include radioisotopes such as barium or cesium, which emit detectable radiation but are not overtly harmful to the subject. Suitable markers for NMR and ESR include those with a detectable characteristic spin, such as deuterium, which may be incorporated into the antibody by labeling of nutrients for the relevant hybridoma.

A TNFR-specific antibody or antibody fragment which has been labeled with an appropriate detectable imaging moiety, such as a radioisotope (for example, ^{131}I , ^{112}In , $^{99\text{m}}\text{Tc}$), a radio-opaque substance, or a material detectable by nuclear magnetic resonance, is introduced (for example, parenterally, subcutaneously or intraperitoneally) into the mammal to be examined for immune system disorder. It will be understood in the art that the size of the subject and the imaging system used will determine the quantity of imaging moiety needed to produce diagnostic images. In the case of a radioisotope moiety, for a human subject, the quantity of radioactivity injected will normally range from about 5 to 20 millicuries of $^{99\text{m}}\text{Tc}$. The labeled antibody or antibody fragment will then preferentially accumulate at the location of cells which contain TNFR protein. *In vivo* tumor imaging is described in S.W. Burchiel et al., "Immunopharmacokinetics of Radiolabeled Antibodies and Their Fragments" (Chapter 13 in *Tumor Imaging: The Radiochemical Detection of*

Cancer, S.W. Burchiel and B. A. Rhodes, eds., Masson Publishing Inc. (1982)).

Treatment

5 The Tumor Necrosis Factor (TNF) family ligands are known to be among the most pleiotropic cytokines, inducing a large number of cellular responses, including cytotoxicity, anti-viral activity, immunoregulatory activities, and the transcriptional regulation of several genes (Goeddel, D.V. *et al.*, "Tumor Necrosis Factors: Gene Structure and Biological Activities," *Symp. Quant. Biol.* 51:597-609 (1986), Cold Spring Harbor; Beutler, B., and Cerami, A., *Annu. Rev. Biochem.* 57:505-518 (1988); Old, L.J., *Sci. Am.* 258:59-75 (1988); Fiers, W., *FEBS Lett.* 285:199-224 (1991)). The TNF-family ligands induce such various cellular responses by binding to TNF-family receptors. Cells which express a TNFR polypeptide and have a potent cellular response to TNFR-6 α and -6 β ligands include lymphocytes, endothelial cells, keratinocytes, and prostate tissue. By "a cellular response to a TNF-family ligand" is intended any genotypic, phenotypic, and/or morphologic change to a cell, cell line, tissue, tissue culture or patient that is induced by a TNF-family ligand. As indicated, such cellular responses include not only normal physiological responses to TNF-family ligands, but also diseases associated with increased apoptosis or the inhibition of apoptosis.

15 Diseases associated with increased cell survival, or the inhibition of apoptosis, include cancers (such as follicular lymphomas, carcinomas with p53 mutations, and hormone-dependent tumors, such as breast cancer, prostate cancer, Kaposi's sarcoma and ovarian cancer); autoimmune disorders (such as systemic lupus erythematosus and immune-related glomerulonephritis rheumatoid arthritis) and viral infections (such as herpes viruses, pox viruses and adenoviruses), information graft v. host disease, acute graft rejection, and chronic graft rejection. Diseases associated with increased apoptosis include AIDS; neurodegenerative disorders (such as Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis, Retinitis pigmentosa, Cerebellar degeneration); myelodysplastic syndromes (such as aplastic anemia), ischemic injury (such as that caused by myocardial infarction, stroke and reperfusion injury), toxin-induced liver disease (such as that caused by alcohol), septic shock, cachexia and anorexia.

25 Thus, in one aspect, the present invention is directed to a method for enhancing apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the TNFR polypeptide an effective

amount of TNFR polypeptide, analog or an agonist capable of increasing TNFR mediated signaling. Preferably, TNFR mediated signaling is increased to treat a disease wherein decreased apoptosis is exhibited. Antagonist can include soluble forms of TNFR and monoclonal antibodies directed against the TNFR polypeptide.

By "agonist" is intended naturally occurring and synthetic compounds capable of enhancing or potentiating apoptosis. By "antagonist" is intended naturally occurring and synthetic compounds capable of inhibiting apoptosis. Whether any candidate "agonist" or "antagonist" of the present invention can enhance or inhibit apoptosis can be determined using art-known TNF-family ligand/receptor cellular response assays, including those described in more detail below.

One such screening procedure involves the use of melanophores which are transfected to express the receptor of the present invention. Such a screening technique is described in PCT WO 92/01810, published February 6, 1992. Such an assay may be employed, for example, for screening for a compound which inhibits (or enhances) activation of the receptor polypeptide of the present invention by contacting the melanophore cells which encode the receptor with both a TNF-family ligand and the candidate antagonist (or agonist). Inhibition or enhancement of the signal generated by the ligand indicates that the compound is an antagonist or agonist of the ligand/receptor signaling pathway.

Other screening techniques include the use of cells which express the receptor (for example, transfected CHO cells) in a system which measures extracellular pH changes caused by receptor activation, for example, as described in *Science* 246:181-296 (October 1989). For example, compounds may be contacted with a cell which expresses the receptor polypeptide of the present invention and a second messenger response, e.g., signal transduction or pH changes, may be measured to determine whether the potential compound activates or inhibits the receptor.

Another such screening technique involves introducing RNA encoding the receptor into *Xenopus* oocytes to transiently express the receptor. The receptor oocytes may then be contacted with the receptor ligand and a compound to be screened, followed by detection of inhibition or activation of a calcium signal in the case of screening for compounds which are thought to inhibit activation of the receptor.

Another screening technique involves expressing in cells a construct wherein the receptor is linked to a phospholipase C or D. Such cells include

endothelial cells, smooth muscle cells, embryonic kidney cells, etc. The screening may be accomplished as hereinabove described by detecting activation of the receptor or inhibition of activation of the receptor from the phospholipase signal.

5 Another method involves screening for compounds which inhibit activation of the receptor polypeptide of the present invention antagonists by determining inhibition of binding of labeled ligand to cells which have the receptor on the surface thereof. Such a method involves transfecting a eukaryotic cell with DNA encoding the receptor such that the cell expresses the
10 receptor on its surface and contacting the cell with a compound in the presence of a labeled form of a known ligand. The ligand can be labeled, e.g., by radioactivity. The amount of labeled ligand bound to the receptors is measured, e.g., by measuring radioactivity of the receptors. If the compound binds to the receptor as determined by a reduction of labeled ligand which binds to the
15 receptors, the binding of labeled ligand to the receptor is inhibited.

Further screening assays for agonist and antagonist of the present invention are described in Tartaglia, L.A., and Goeddel, D.V., *J. Biol. Chem.* 267(7):4304-4307(1992).

20 Thus, in a further aspect, a screening method is provided for determining whether a candidate agonist or antagonist is capable of enhancing or inhibiting a cellular response to a TNF-family ligand. The method involves contacting cells which express the TNFR polypeptide with a candidate compound and a TNF-family ligand, assaying a cellular response, and comparing the cellular response to a standard cellular response, the standard
25 being assayed when contact is made with the ligand in absence of the candidate compound, whereby an increased cellular response over the standard indicates that the candidate compound is an agonist of the ligand/receptor signaling pathway and a decreased cellular response compared to the standard indicates that the candidate compound is an antagonist of the ligand/receptor signaling
30 pathway. By "assaying a cellular response" is intended qualitatively or quantitatively measuring a cellular response to a candidate compound and/or a TNF-family ligand (e.g., determining or estimating an increase or decrease in T cell proliferation or tritiated thymidine labeling). By the invention, a cell expressing the TNFR polypeptide can be contacted with either an endogenous
35 or exogenously administered TNF-family ligand.

Agonist according to the present invention include naturally occurring and synthetic compounds such as, for example, TNF family ligand peptide fragments, transforming growth factor, neurotransmitters (such as glutamate,

dopamine, *N*-methyl-D-aspartate), tumor suppressors (p53), cytolytic T cells and antimetabolites. Preferred agonist include chemotherapeutic drugs such as, for example, cisplatin, doxorubicin, bleomycin, cytosine arabinoside, nitrogen mustard, methotrexate and vincristine. Others include ethanol and β -amyloid peptide. (Science 267:1457-1458 (1995)). Further preferred agonist include polyclonal and monoclonal antibodies raised against the TNFR polypeptide, or a fragment thereof. Such agonist antibodies raised against a TNF-family receptor are disclosed in Tartaglia, L.A., *et al.*, *Proc. Natl. Acad. Sci. USA* 88:9292-9296 (1991); and Tartaglia, L.A., and Goeddel, D.V., *J. Biol. Chem.* 267 (7):4304-4307 (1992) See, also, PCT Application WO 94/09137.

Antagonist according to the present invention include naturally occurring and synthetic compounds such as, for example, the CD40 ligand, neutral amino acids, zinc, estrogen, androgens, viral genes (such as Adenovirus *E1B*, Baculovirus *p35* and *IAP*, Cowpox virus *crmA*, Epstein-Barr virus *BHRF1*, *LMP-1*, African swine fever virus *LMW5-HL*, and Herpesvirus *yl 34.5*), calpain inhibitors, cysteine protease inhibitors, and tumor promoters (such as PMA, Phenobarbital, and γ -Hexachlorocyclohexane). Other antagonists include polyclonal and monoclonal antagonist antibodies raised against the TNFR polypeptides or a fragment thereof. Such antagonist antibodies raised against a TNF-family receptor are described in Tartaglia, L.A., and Goeddel, D.V., *J. Biol. Chem.* 267(7):4304-4307 (1992) and Tartaglia, L.A. *et al.*, *Cell* 73:213-216 (1993). See, also, PCT Application WO 94/09137.

Other potential antagonists include antisense molecules. Antisense technology can be used to control gene expression through antisense DNA or RNA or through triple-helix formation. Antisense techniques are discussed, for example, in Okano, *J. Neurochem.* 56:560 (1991); *Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression*, CRC Press, Boca Raton, FL (1988). Triple helix formation is discussed in, for instance Lee *et al.*, *Nucleic Acids Research* 6:3073 (1979); Cooney *et al.*, *Science* 241:456 (1988); and Dervan *et al.*, *Science* 251:1360 (1991). The methods are based on binding of a polynucleotide to a complementary DNA or RNA.

For example, the 5' coding portion of a polynucleotide that encodes the mature polypeptide of the present invention may be used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription thereby preventing transcription and the production of the receptor. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into receptor polypeptide.

The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of the receptor.

Further antagonist according to the present invention include soluble forms of TNFR, i.e., TNFR fragments that include the ligand binding domain from the extracellular region of the full length receptor. Such soluble forms of the receptor, which may be naturally occurring or synthetic, antagonize TNFR mediated signaling by competing with the cell surface TNFR for binding to TNF-family ligands. Thus, soluble forms of the receptor that include the ligand binding domain are novel cytokines capable of inhibiting tumor necrosis induced by TNF-family ligands. Other such cytokines are known in the art and include Fas B (a soluble form of the mouse Fas receptor) that acts physiologically to limit apoptosis induced by Fas ligand (Hughes, D.P. and Crispe, I.N., *J. Exp. Med.* 182:1395-1401 (1995)).

As indicated polyclonal and monoclonal antibody agonist or antagonist according to the present invention can be raised according to the methods disclosed in Tartaglia, L.A., and Goeddel, D.V., *J. Biol. Chem.* 267(7):4304-4307(1992); Tartaglia, L.A. *et al.*, *Cell* 73:213-216 (1993), and PCT Application WO 94/09137. The term "antibody" (Ab) or "monoclonal antibody" (mAb) as used herein is meant to include intact molecules as well as fragments thereof (such as, for example, Fab and F(ab')₂ fragments) which are capable of binding an antigen. Fab and F(ab')₂ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl *et al.*, *J. Nucl. Med.* 24:316-325 (1983)).

Antibodies according to the present invention may be prepared by any of a variety of methods described above, and known in the art

Proteins and other compounds which bind the extracellular domains are also candidate agonist and antagonist according to the present invention. Such binding compounds can be "captured" using the yeast two-hybrid system (Fields and Song, *Nature* 340:245-246 (1989)). A modified version of the yeast two-hybrid system has been described by Roger Brent and his colleagues (Gyuris, J. *et al.*, *Cell* 75:791-803 (1993); Zervos, A.S. *et al.*, *Cell* 72:223-232 (1993)).

By a "TNF-family ligand" is intended naturally occurring, recombinant, and synthetic ligands that are capable of binding to a member of the TNF receptor family and inducing the ligand/receptor signaling pathway. Members of the TNF ligand family include, but are not limited to, the TNFR-6 α & -6 β

ligands, TNF- α , lymphdotoxin- α (LT- α , also known as TNF- β), LT- β , FasL, CD40, CD27, CD30, 4-1BB, OX40, TRAIL and nerve growth factor (NGF).

Representative therapeutic applications of the present invention are discussed in more detail below. The state of immunodeficiency that defines AIDS is secondary to a decrease in the number and function of CD4⁺ T-lymphocytes. Recent reports estimate the daily loss of CD4⁺ T cells to be between 3.5×10^7 and 2×10^9 cells (Wei X., *et al.*, *Nature* 373:117-122 (1995)). One cause of CD4⁺ T cell depletion in the setting of HIV infection is believed to be HIV-induced apoptosis. Indeed, HIV-induced apoptotic cell death has been demonstrated not only *in vitro* but also, more importantly, in infected individuals (Ameisen, J.C., *AIDS* 8:1197-1213 (1994); Finkel, T.H., and Banda, N.K., *Curr. Opin. Immunol.* 6:605-615(1995); Muro-Cacho, C.A. *et al.*, *J. Immunol.* 154:5555-5566 (1995)). Furthermore, apoptosis and CD4⁺ T-lymphocyte depletion is tightly correlated in different animal models of AIDS (Brunner, T., *et al.*, *Nature* 373:441-444 (1995); Gougeon, M.L., *et al.*, *AIDS Res. Hum. Retroviruses* 9:553-563 (1993)) and, apoptosis is not observed in those animal models in which viral replication does not result in AIDS (Gougeon, M.L. *et al.*, *AIDS Res. Hum. Retroviruses* 9:553-563 (1993)). Further data indicates that uninfected but primed or activated T lymphocytes from HIV-infected individuals undergo apoptosis after encountering the TNF-family ligand FasL. Using monocytic cell lines that result in death following HIV infection, it has been demonstrated that infection of U937 cells with HIV results in the *de novo* expression of FasL and that FasL mediates HIV-induced apoptosis (Badley, A.D. *et al.*, *J. Virol.* 70:199-206 (1996)). Further the TNF-family ligand was detectable in uninfected macrophages and its expression was upregulated following HIV infection resulting in selective killing of uninfected CD4 T-lymphocytes (Badley, A.D. *et al.*, *J. Virol.* 70:199-206 (1996)). Thus, by the invention, a method for treating HIV⁺ individuals is provided which involves administering an antagonist of the present invention to reduce selective killing of CD4 T-lymphocytes. Modes of administration and dosages are discussed in detail below.

In rejection of an allograft, the immune system of the recipient animal has not previously been primed to respond because the immune system for the most part is only primed by environmental antigens. Tissues from other members of the same species have not been presented in the same way that, for example, viruses and bacteria have been presented. In the case of allograft rejection, immunosuppressive regimens are designed to prevent the immune

system from reaching the effector stage. However, the immune profile of xenograft rejection may resemble disease recurrence more than allograft rejection. In the case of disease recurrence, the immune system has already been activated, as evidenced by destruction of the native islet cells. Therefore, in disease recurrence the immune system is already at the effector stage. Agonist of the present invention are able to suppress the immune response to both allografts and xenografts because lymphocytes activated and differentiated into effector cells will express the TNFR polypeptide, and thereby are susceptible to compounds which enhance TNFR activity. Thus, the present invention further provides a method for creating immune privileged tissues. Antagonist of the invention can further be used in the treatment of Inflammatory Bowel-Disease.

Formulations

The TNFR polypeptide composition will be formulated and dosed in a fashion consistent with good medical practice, taking into account the clinical condition of the individual patient (especially the side effects of treatment with TNFR-6 α or -6 β polypeptide alone), the site of delivery of the TNFR polypeptide composition, the method of administration, the scheduling of administration, and other factors known to practitioners. The "effective amount" of TNFR polypeptide for purposes herein is thus determined by such considerations.

As a general proposition, the total pharmaceutically effective amount of TNFR polypeptide administered parenterally per dose will be in the range of about 1 μ g/kg/day to 10 mg/kg/day of patient body weight, although, as noted above, this will be subject to therapeutic discretion. More preferably, this dose is at least 0.01 mg/kg/day, and most preferably for humans between about 0.01 and 1 mg/kg/day for the hormone. If given continuously, the TNFR polypeptide is typically administered at a dose rate of about 1 μ g/kg/hour to about 50 μ g/kg/hour, either by 1-4 injections per day or by continuous subcutaneous infusions, for example, using a mini-pump. An intravenous bag solution may also be employed. The length of treatment needed to observe changes and the interval following treatment for responses to occur appears to vary depending on the desired effect.

Pharmaceutical compositions containing the TNFR of the invention may be administered orally, rectally, parenterally, intracisternally, intravaginally, intraperitoneally, topically (as by powders, ointments, drops or transdermal patch), buccally, or as an oral or nasal spray. By "pharmaceutically acceptable

carrier" is meant a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

The TNFR polypeptide is also suitably administered by sustained-release systems. Suitable examples of sustained-release compositions include semi-permeable polymer matrices in the form of shaped articles, e.g., films, or microcapsules. Sustained-release matrices include polylactides (U.S. Pat. No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma-ethyl-L-glutamate (Sidman, U. et al., *Biopolymers* 22:547-556 (1983)), poly (2-hydroxyethyl methacrylate) (R. Langer et al., *J. Biomed. Mater. Res.* 15:167-277 (1981), and R. Langer, *Chem. Tech.* 12:98-105 (1982)), ethylene vinyl acetate (R. Langer et al., *Id.*) or poly-D- (-)-3-hydroxybutyric acid (EP 133,988). Sustained-release TNFR polypeptide compositions also include liposomally entrapped TNFR polypeptides. Liposomes containing TNFR polypeptides are prepared by methods known per se: DE 3,218,121; Epstein et al., *Proc. Natl. Acad. Sci. (USA)* 82:3688-3692 (1985); Hwang et al., *Proc. Natl. Acad. Sci. (USA)* 77:4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese Pat. Appl. 83-118008; U.S. Pat. Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily, the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. percent cholesterol, the selected proportion being adjusted for the optimal TNFR polypeptide therapy.

For parenteral administration, in one embodiment, the TNFR polypeptide is formulated generally by mixing it at the desired degree of purity, in a unit dosage injectable form (solution, suspension, or emulsion), with a pharmaceutically acceptable carrier, i.e., one that is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation. For example, the formulation preferably does not include oxidizing agents and other compounds that are known to be deleterious to polypeptides.

Generally, the formulations are prepared by contacting the TNFR polypeptide uniformly and intimately with liquid carriers or finely divided solid carriers or both. Then, if necessary, the product is shaped into the desired formulation. Preferably the carrier is a parenteral carrier, more preferably a solution that is isotonic with the blood of the recipient. Examples of such carrier vehicles include water, saline, Ringer's solution, and dextrose solution.

Non-aqueous vehicles such as fixed oils and ethyl oleate are also useful herein, as well as liposomes.

The carrier suitably contains minor amounts of additives such as substances that enhance isotonicity and chemical stability. Such materials are non-toxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, succinate, acetic acid, and other organic acids or their salts; antioxidants such as ascorbic acid; low molecular weight (less than about ten residues) polypeptides, e.g., polyarginine or tripeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids, such as glycine, glutamic acid, aspartic acid, or arginine; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, manose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; counterions such as sodium; and/or nonionic surfactants such as polysorbates, poloxamers, or PEG.

The TNFR polypeptide is typically formulated in such vehicles at a concentration of about 0.1 mg/ml to 100 mg/ml, preferably 1-10 mg/ml, at a pH of about 3 to 8. It will be understood that the use of certain of the foregoing excipients, carriers, or stabilizers will result in the formation of TNFR polypeptide salts.

TNFR polypeptides to be used for therapeutic administration must be sterile. Sterility is readily accomplished by filtration through sterile filtration membranes (e.g., 0.2 micron membranes). Therapeutic TNFR polypeptide compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

TNFR polypeptides ordinarily will be stored in unit or multi-dose containers, for example, sealed ampoules or vials, as an aqueous solution or as a lyophilized formulation for reconstitution. As an example of a lyophilized formulation, 10-ml vials are filled with 5 ml of sterile-filtered 1% (w/v) aqueous TNFR polypeptide solution, and the resulting mixture is lyophilized. The infusion solution is prepared by reconstituting the lyophilized TNFR polypeptide using bacteriostatic Water-for-Injection.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human

administration. In addition, the polypeptides of the present invention may be employed in conjunction with other therapeutic compounds.

Chromosome Assays

5 The nucleic acid molecules of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

10 In certain preferred embodiments in this regard, the cDNAs herein disclosed are used to clone genomic DNA of a TNFR protein gene. This can be accomplished using a variety of well known techniques and libraries, which generally are available commercially. The genomic DNA then is used for *in situ* chromosome mapping using well known techniques for this purpose.

15 In addition, in some cases, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the 3' untranslated region of the gene is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Fluorescence *in situ* hybridization ("FISH") of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with probes from the cDNA as short as 50 or 60 bp. For a review of this technique, see Verma *et al.*, *Human Chromosomes: A Manual Of Basic Techniques*, Pergamon Press, New York (1988).

20 25 30 Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, *Mendelian Inheritance In Man*, available on-line through Johns Hopkins University, Welch Medical Library. The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

35 Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is

observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

Examples

*Example 1: Expression and Purification of TNFR-6 α and -6 β in *E. coli**

The bacterial expression vector pQE60 is used for bacterial expression in this example (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311). pQE60 encodes ampicillin antibiotic resistance ("Ampr") and contains a bacterial origin of replication ("ori"), an IPTG inducible promoter, a ribosome binding site ("RBS"), six codons encoding histidine residues that allow affinity purification using nickel-nitrilo-tri-acetic acid ("Ni-NTA") affinity resin sold by QIAGEN, Inc., *supra*, and suitable single restriction enzyme cleavage sites. These elements are arranged such that a DNA fragment encoding a polypeptide may be inserted in such a way as to produce that polypeptide with the six His residues (i.e., a "6 X His tag") covalently linked to the carboxyl terminus of that polypeptide. However, in this example, the polypeptide coding sequence is inserted such that translation of the six His codons is prevented and, therefore, the polypeptide is produced with no 6 X His tag.

The DNA sequences encoding the desired portions of TNFR-6 α and -6 β proteins comprising the mature forms of the TNFR-6 α and -6 β amino acid sequences are amplified from the deposited cDNA clones using PCR oligonucleotide primers which anneal to the amino terminal sequences of the desired portions of the TNFR-6 α or -6 β proteins and to sequences in the deposited constructs 3' to the cDNA coding sequence. Additional nucleotides containing restriction sites to facilitate cloning in the pQE60 vector are added to the 5' and 3' sequences, respectively.

For cloning the mature form of the TNFR-6 α protein, the 5' primer has the sequence 5' CGCCCATGGCGAGAAACACCCACCTAC 3' (SEQ ID NO:19) containing the underlined NcoI restriction site. One of ordinary skill in the art would appreciate, of course, that the point in the protein coding sequence where the 5' primer begins may be varied to amplify a desired portion of the

complete protein shorter or longer than the mature form. The 3' primer has the sequence 5' CGCAAGCTTCTCTTTTCAGTGCAAGTG 3' (SEQ ID NO:20) containing the underlined HindIII restriction site. For cloning the mature form of the TNFR-6 β protein, the 5' primer has the sequence of SEQ ID NO:19 above, and the 3' primer has the sequence 5' CGCAAGCTTCTCCTCAGCTCCTGCAGTG 3' (SEQ ID NO:21) containing the underlined HindIII restriction site.

The amplified TNFR-6 α and -6 β DNA fragments and the vector pQE60 are digested with NcoI and HindIII and the digested DNAs are then ligated together. Insertion of the TNFR-6 α and -6 β DNA into the restricted pQE60 vector places the TNFR-6 α and -6 β protein coding region including its associated stop codon downstream from the IPTG-inducible promoter and in-frame with an initiating AUG. The associated stop codon prevents translation of the six histidine codons downstream of the insertion point.

The ligation mixture is transformed into competent *E. coli* cells using standard procedures such as those described in Sambrook et al., *Molecular Cloning: a Laboratory Manual, 2nd Ed.*; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989). *E. coli* strain M15/rep4, containing multiple copies of the plasmid pREP4, which expresses the lac repressor and confers kanamycin resistance ("Kan^r"), is used in carrying out the illustrative example described herein. This strain, which is only one of many that are suitable for expressing TNFR-6 α or -6 β protein, is available commercially from QIAGEN, Inc., *supra*. Transformants are identified by their ability to grow on LB plates in the presence of ampicillin and kanamycin. Plasmid DNA is isolated from resistant colonies and the identity of the cloned DNA confirmed by restriction analysis, PCR and DNA sequencing.

Clones containing the desired constructs are grown overnight ("O/N") in liquid culture in LB media supplemented with both ampicillin (100 μ g/ml) and kanamycin (25 μ g/ml). The O/N culture is used to inoculate a large culture, at a dilution of approximately 1:25 to 1:250. The cells are grown to an optical density at 600 nm ("OD₆₀₀") of between 0.4 and 0.6. isopropyl-b-D-thiogalactopyranoside ("IPTG") is then added to a final concentration of 1 mM to induce transcription from the *lac* repressor sensitive promoter, by inactivating the lacI repressor. Cells subsequently are incubated further for 3 to 4 hours. Cells then are harvested by centrifugation.

To purify the TNFR-6 α and -6 β polypeptide, the cells are then stirred for 3-4 hours at 4° C in 6M guanidine-HCl, pH 8. The cell debris is removed by centrifugation, and the supernatant containing the TNFR-6 α and -6 β is dialyzed against 50 mM Na-acetate buffer pH 6, supplemented with 200 mM NaCl. Alternatively, the protein can be successfully refolded by dialyzing it against 500 mM NaCl, 20% glycerol, 25 mM Tris/HCl pH 7.4, containing protease inhibitors. After renaturation the protein can be purified by ion exchange, hydrophobic interaction and size exclusion chromatography. Alternatively, an affinity chromatography step such as an antibody column can be used to obtain pure TNFR-6 α and -6 β protein. The purified protein is stored at 4° C or frozen at -80° C.

The following alternative method may be used to purify TNFR-6 α or -6 β expressed in *E coli* when it is present in the form of inclusion bodies.

Unless otherwise specified, all of the following steps are conducted at 4-10°C.

Upon completion of the production phase of the *E. coli* fermentation, the cell culture is cooled to 4-10°C and the cells are harvested by continuous centrifugation at 15,000 rpm (Heraeus Sepatech). On the basis of the expected yield of protein per unit weight of cell paste and the amount of purified protein required, an appropriate amount of cell paste, by weight, is suspended in a buffer solution containing 100 mM Tris, 50 mM EDTA, pH 7.4. The cells are dispersed to a homogeneous suspension using a high shear mixer.

The cells were then lysed by passing the solution through a microfluidizer (Microfluidics, Corp. or APV Gaulin, Inc.) twice at 4000-6000 psi. The homogenate is then mixed with NaCl solution to a final concentration of 0.5 M NaCl, followed by centrifugation at 7000 xg for 15 min. The resultant pellet is washed again using 0.5M NaCl, 100 mM Tris, 50 mM EDTA, pH 7.4.

The resulting washed inclusion bodies are solubilized with 1.5 M guanidine hydrochloride (GuHCl) for 2-4 hours. After 7000 xg centrifugation for 15 min., the pellet is discarded and the TNFR-6 α or -6 β

polypeptide-containing supernatant is incubated at 4°C overnight to allow further GuHCl extraction.

Following high speed centrifugation (30,000 xg) to remove insoluble particles, the GuHCl solubilized protein is refolded by quickly mixing the

GuHCl extract with 20 volumes of buffer containing 50 mM sodium, pH 4.5, 150 mM NaCl, 2 mM EDTA by vigorous stirring. The refolded diluted protein solution is kept at 4°C without mixing for 12 hours prior to further purification steps.

5 To clarify the refolded TNF receptor polypeptide solution, a previously prepared tangential filtration unit equipped with 0.16 µm membrane filter with appropriate surface area (e.g., Filtron), equilibrated with 40 mM sodium acetate, pH 6.0 is employed. The filtered sample is loaded onto a cation exchange resin (e.g., Poros HS-50, Perseptive Biosystems). The column is washed with 40 mM sodium acetate, pH 6.0 and eluted with 250 mM, 500 mM, 1000 mM, and 1500 mM NaCl in the same buffer, in a stepwise manner. The absorbance at 280 nm of the effluent is continuously monitored. Fractions are collected and further analyzed by SDS-PAGE.

15 Fractions containing the TNF receptor polypeptide are then pooled and mixed with 4 volumes of water. The diluted sample is then loaded onto a previously prepared set of tandem columns of strong anion (Poros HQ-50, Perseptive Biosystems) and weak anion (Poros CM-20, Perseptive Biosystems) exchange resins. The columns are equilibrated with 40 mM sodium acetate, pH 6.0. Both columns are washed with 40 mM sodium acetate, pH 6.0, 200 mM NaCl. The CM-20 column is then eluted using a 10 column volume linear gradient ranging from 0.2 M NaCl, 50 mM sodium acetate, pH 6.0 to 1.0 M NaCl, 50 mM sodium acetate, pH 6.5. Fractions are collected under constant A_{280} monitoring of the effluent. Fractions containing the TNFR-6α or -6β polypeptide (determined, for instance, by 16% SDS-PAGE) are then pooled.

25 The resultant TNF receptor polypeptide exhibits greater than 95% purity after the above refolding and purification steps. No major contaminant bands are observed from Commassie blue stained 16% SDS-PAGE gel when 5 µg of purified protein is loaded. The purified protein is also tested for endotoxin/LPS contamination, and typically the LPS content is less than 0.1 ng/ml according to LAL assays.

Example 2: Cloning and Expression of TNFR-6α and -6β proteins in a Baculovirus Expression System

In this illustrative example, the plasmid shuttle vector pA2 is used to insert the cloned DNA encoding complete protein, including its naturally

associated secretory signal (leader) sequence, into a baculovirus to express the mature TNFR-6 α or -6 β protein, using standard methods as described in Summers *et al.*, *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Agricultural Experimental Station Bulletin No. 1555 (1987). This expression vector contains the strong polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) followed by convenient restriction sites such as BamHI, Xba I and Asp718. The polyadenylation site of the simian virus 40 ("SV40") is used for efficient polyadenylation. For easy selection of recombinant virus, the plasmid contains the beta-galactosidase gene from *E. coli* under control of a weak *Drosophila* promoter in the same orientation, followed by the polyadenylation signal of the polyhedrin gene. The inserted genes are flanked on both sides by viral sequences for cell-mediated homologous recombination with wild-type viral DNA to generate a viable virus that express the cloned polynucleotide.

Many other baculovirus vectors could be used in place of the vector above, such as pAc373, pVL941 and pAcIM1, as one skilled in the art would readily appreciate, as long as the construct provides appropriately located signals for transcription, translation, secretion and the like, including a signal peptide and an in-frame AUG as required. Such vectors are described, for instance, in Luckow *et al.*, *Virology* 170:31-39 (1989).

The cDNA sequence encoding the full length TNFR-6 α or -6 β protein in a deposited clone, including the AUG initiation codon and the naturally associated leader sequence shown in SEQ ID NO:2 or 4 is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene.

The 5' primer for TNFR-6 α and -6 β has the sequence

5' CGCGGATCCGCCATCATGAGGGCGTGGAGGGGCCAG 3' (SEQ ID NO:24) containing the underlined BamHI restriction enzyme site. All of the previously described primers encode an efficient signal for initiation of translation in eukaryotic cells, as described by Kozak, M., *J. Mol. Biol.*

196:947-950 (1987). The 3' primer for TNFR-6 α has the sequence

5' CGCGGTACCCTCTTTCAGTGCAAGTG 3' (SEQ ID NO:25) containing the underlined Asp718 restriction site. The 3' primer for TNFR-6 β has the sequence 5' CGCGGTACCCTCCTCAGCTCCTGCAGTG 3' (SEQ ID NO:27) containing the underlined Asp718 restriction site.

The amplified fragment is isolated from a 1% agarose gel using a commercially available kit ("GeneClean," BIO 101 Inc., La Jolla, Ca.). The fragment then is digested with the appropriate restriction enzyme for each of the primers used, as specified above, and again is purified on a 1% agarose gel.

5 The plasmid is digested with the same restriction enzymes and optionally, can be dephosphorylated using calf intestinal phosphatase, using routine procedures known in the art. The DNA is then isolated from a 1% agarose gel using a commercially available kit ("GeneClean" BIO 101 Inc., La Jolla, Ca.).

10 The fragment and dephosphorylated plasmid are ligated together with T4 DNA ligase. *E. coli* HB101 or other suitable *E. coli* hosts such as XL-1 Blue (Statagene Cloning Systems, La Jolla, CA) cells are transformed with the ligation mixture and spread on culture plates. Bacteria are identified that contain the plasmid with the human TNF receptor gene by digesting DNA from
15 individual colonies using the enzymes used immediately above and then analyzing the digestion product by gel electrophoresis. The sequence of the cloned fragment is confirmed by DNA sequencing. This plasmid is designated herein pA2-TNFR-6 α or pA2TNFR-6 β (collectively pA2-TNFR).

20 Five μ g of the plasmid pA2-TNFR is co-transfected with 1.0 μ g of a commercially available linearized baculovirus DNA ("BaculoGold™ baculovirus DNA", Pharmingen, San Diego, CA), using the lipofection method described by Felgner et al., *Proc. Natl. Acad. Sci. USA* 84: 7413-7417 (1987). One μ g of BaculoGold™ virus DNA and 5 μ g of the plasmid pA2-TNFR are mixed in a sterile well of a microtiter plate containing 50 μ l of serum-free Grace's medium.
25 (Life Technologies Inc., Gaithersburg, MD). Afterwards, 10 μ l Lipofectin plus 90 μ l Grace's medium are added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture is added drop-wise to Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate is then incubated for 5 hours at 27°
30 C. The transfection solution is then removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum is added. Cultivation is then continued at 27° C for four days.

After four days the supernatant is collected and a plaque assay is performed, as described by Summers and Smith, *supra*. An agarose gel with
35 "Blue Gal" (Life Technologies Inc., Gaithersburg) is used to allow easy identification and isolation of gal-expressing clones, which produce blue-stained

plaques. (A detailed description of a "plaque assay" of this type can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10). After appropriate incubation, blue stained plaques are picked with the tip of a micropipettor (e.g., Eppendorf). The agar containing the recombinant viruses is then resuspended in a microcentrifuge tube containing 200 μ l of Grace's medium and the suspension containing the recombinant baculovirus is used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes are harvested and then they are stored at 4° C.

To verify the expression of the TNF receptor gene Sf9 cells are grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells are infected with the recombinant baculovirus at a multiplicity of infection ("MOI") of about 2. If radiolabeled proteins are desired, 6 hours later the medium is removed and is replaced with SF900 II medium minus methionine and cysteine (available from Life Technologies Inc., Rockville, MD). After 42 hours, 5 μ Ci of 35 S-methionine and 5 μ Ci 35 S-cysteine (available from Amersham) are added. The cells are further incubated for 16 hours and then are harvested by centrifugation. The proteins in the supernatant as well as the intracellular proteins are analyzed by SDS-PAGE followed by autoradiography (if radiolabeled).

Microsequencing of the amino acid sequence of the amino terminus of purified protein may be used to determine the amino terminal sequence of the mature form of the TNF receptor protein.

Example 3: Cloning and Expression of TNFR-6 α and -6 β in Mammalian Cells

A typical mammalian expression vector contains the promoter element, which mediates the initiation of transcription of mRNA, the protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRs) from Retroviruses, e.g., RSV, HTLV1, HIV1 and the early promoter of the cytomegalovirus (CMV). However, cellular elements can also be used (e.g., the human actin promoter). Suitable expression vectors for use in practicing the present invention include, for example, vectors such as pSVL and pMSG

(Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146) and pBC12MI (ATCC 67109). Mammalian host cells that could be used include, human Hela, 293, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV1, quail QC1-3 cells, mouse L cells and Chinese hamster ovary (CHO) cells.

Alternatively, the gene can be expressed in stable cell lines that contain the gene integrated into a chromosome. The co-transfection with a selectable marker such as dhfr, gpt, neomycin, hygromycin allows the identification and isolation of the transfected cells.

The transfected gene can also be amplified to express large amounts of the encoded protein. The DHFR (dihydrofolate reductase) marker is useful to develop cell lines that carry several hundred or even several thousand copies of the gene of interest. Another useful selection marker is the enzyme glutamine synthase (GS) (Murphy et al., *Biochem J.* 227:277-279 (1991); Bebbington et al., *Bio/Technology* 10:169-175 (1992)). Using these markers, the mammalian cells are grown in selective medium and the cells with the highest resistance are selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) and NSO cells are often used for the production of proteins.

The expression vectors pC1 and pC4 contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen et al., *Molecular and Cellular Biology*, 438-447 (March, 1985)) plus a fragment of the CMV-enhancer (Boshart et al., *Cell* 41:521-530 (1985)). Multiple cloning sites, e.g., with the restriction enzyme cleavage sites BamHI, XbaI and Asp718, facilitate the cloning of the gene of interest. The vectors contain in addition the 3' intron, the polyadenylation and termination signal of the rat preproinsulin gene.

Example 3(a): Cloning and Expression in COS Cells

The expression plasmid, pTNFR- α -HA and - β -HA, is made by cloning a portion of the cDNA encoding the mature form of the TNF receptor protein into the expression vector pcDNAI/Amp or pcDNAIII (which can be obtained from Invitrogen, Inc.).

The expression vector pcDNAI/amp contains: (1) an *E. coli* origin of replication effective for propagation in *E. coli* and other prokaryotic cells; (2) an ampicillin resistance gene for selection of plasmid-containing prokaryotic cells; (3) an SV40 origin of replication for propagation in eukaryotic cells; (4) a CMV promoter, a polylinker, an SV40 intron; (5) several codons encoding a

hemagglutinin fragment (i.e., an "HA" tag to facilitate purification) followed by a termination codon and polyadenylation signal arranged so that a cDNA can be conveniently placed under expression control of the CMV promoter and operably linked to the SV40 intron and the polyadenylation signal by means of restriction sites in the polylinker. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein described by Wilson et al., *Cell* 37: 767 (1984). The fusion of the HA tag to the target protein allows easy detection and recovery of the recombinant protein with an antibody that recognizes the HA epitope. pcDNAIII contains, in addition, the selectable neomycin marker.

A DNA fragment encoding the complete TNF receptor polypeptide is cloned into the polylinker region of the vector so that recombinant protein expression is directed by the CMV promoter. The plasmid construction strategy is as follows. The TNF receptor cDNA of a deposited clone is amplified using primers that contain convenient restriction sites, much as described above for construction of vectors for expression of a TNF receptor in *E. coli*. Suitable primers can easily be designed by those of ordinary skill in the art.

The PCR amplified DNA fragment and the vector, pcDNAI/Amp, are digested with XbaI and EcoRI and then ligated. The ligation mixture is transformed into *E. coli* strain SURE (available from Stratagene Cloning Systems, 11099 North Torrey Pines Road, La Jolla, CA 92037), and the transformed culture is plated on ampicillin media plates which then are incubated to allow growth of ampicillin resistant colonies. Plasmid DNA is isolated from resistant colonies and examined by restriction analysis or other means for the presence of the fragment encoding the TNFR- α and - 6β polypeptides.

For expression of recombinant TNFR- α and - 6β , COS cells are transfected with an expression vector, as described above, using DEAE-DEXTRAN, as described, for instance, in Sambrook et al., *Molecular Cloning: a Laboratory Manual*, Cold Spring Laboratory Press, Cold Spring Harbor, New York (1989). Cells are incubated under conditions for expression of TNFR by the vector.

Expression of the pTNFR- α -HA and - 6β -HA fusion protein is detected by radiolabeling and immunoprecipitation, using methods described in, for example Harlow et al., *Antibodies: A Laboratory Manual, 2nd Ed.*; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1988). To this end, two days after transfection, the cells are labeled by incubation in media

containing ^{35}S -cysteine for 8 hours. The cells and the media are collected, and the cells are washed and lysed with detergent-containing RIPA buffer: 150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50 mM TRIS, pH 7.5, as described by Wilson et al. cited above. Proteins are precipitated from the cell lysate and from the culture media using an HA-specific monoclonal antibody. The precipitated proteins then are analyzed by SDS-PAGE and autoradiography. An expression product of the expected size is seen in the cell lysate, which is not seen in negative controls.

Example 3(b): Cloning and Expression in CHO Cells

The vector pC4 is used for the expression of TNFR-6 α and -6 β polypeptides. Plasmid pC4 is a derivative of the plasmid pSV2-dhfr (ATCC Accession No. 37146). The plasmid contains the mouse DHFR gene under control of the SV40 early promoter. Chinese hamster ovary- or other cells lacking dihydrofolate activity that are transfected with these plasmids can be selected by growing the cells in a selective medium (alpha minus MEM, Life Technologies) supplemented with the chemotherapeutic agent methotrexate. The amplification of the DHFR genes in cells resistant to methotrexate (MTX) has been well documented (see, e.g., Alt, F. W., Kellems, R. M., Bertino, J. R., and Schimke, R. T., 1978, *J. Biol. Chem.* 253:1357-1370, Hamlin, J. L. and Ma, C. 1990, *Biochem. et Biophys. Acta*, 1097:107-143, Page, M. J. and Sydenham, M. A. 1991, *Biotechnology* 9:64-68). Cells grown in increasing concentrations of MTX develop resistance to the drug by overproducing the target enzyme, DHFR, as a result of amplification of the DHFR gene. If a second gene is linked to the DHFR gene, it is usually co-amplified and over-expressed. It is known in the art that this approach may be used to develop cell lines carrying more than 1,000 copies of the amplified gene(s). Subsequently, when the methotrexate is withdrawn, cell lines are obtained which contain the amplified gene integrated into one or more chromosome(s) of the host cell.

Plasmid pC4 contains for expressing the gene of interest the strong promoter of the long terminal repeat (LTR) of the Rouse Sarcoma Virus (Cullen, et al., *Molecular and Cellular Biology*, March 1985:438-447) plus a fragment isolated from the enhancer of the immediate early gene of human cytomegalovirus (CMV) (Boshart et al., *Cell* 41:521-530 (1985)). Downstream of the promoter are the following single restriction enzyme cleavage sites that allow the integration of the genes: BamHI, Xba I, and Asp718. Behind these cloning sites the plasmid contains the 3' intron and polyadenylation site of the

rat preproinsulin gene. Other high efficiency promoters can also be used for the expression, e.g., the human β -actin promoter, the SV40 early or late promoters or the long terminal repeats from other retroviruses, e.g., HIV and HTLV. Clontech's Tet-Off and Tet-On gene expression systems and similar systems can be used to express the TNF receptor polypeptide in a regulated way in mammalian cells (Gossen, M., & Bujard, H. 1992, *Proc. Natl. Acad. Sci. USA* 89:5547-5551). For the polyadenylation of the mRNA other signals, e.g., from the human growth hormone or globin genes can be used as well. Stable cell lines carrying a gene of interest integrated into the chromosomes can also be selected upon co-transfection with a selectable marker such as gpt, G418 or hygromycin. It is advantageous to use more than one selectable marker in the beginning, e.g., G418 plus methotrexate.

The plasmid pC4 is digested with the restriction enzymes appropriate for the specific primers used to amplify the TNF receptor of choice as outlined below and then dephosphorylated using calf intestinal phosphates by procedures known in the art. The vector is then isolated from a 1% agarose gel.

The DNA sequence encoding the TNF receptor polypeptide is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the desired portion of the gene. The 5' primer for TNFR-6 α and -6 β containing the underlined BamHI site, has the following sequence:

5' CGCGGATCCGCCATCATGAGGGCGTGGAGGGGCCAG 3' (SEQ ID NO:25). The 3' primer for TNFR-6 α has the sequence

5' CGCGGTACCCTCTTTCAGTGCAAGTG 3' (SEQ ID NO:26) containing the underlined Asp718 restriction site. The 3' primer for TNFR-6 β has the sequence 5' CGCGGTACCCTCCTCAGCTCCTGCAGTG 3' (SEQ ID NO:27) containing the underlined Asp718 restriction site.

The amplified fragment is digested with the endonucleases which will cut at the engineered restriction site(s) and then purified again on a 1% agarose gel. The isolated fragment and the dephosphorylated vector are then ligated with T4 DNA ligase. *E. coli* HB101 or XL-1 Blue cells are then transformed and bacteria are identified that contain the fragment inserted into plasmid pC4 using, for instance, restriction enzyme analysis.

Chinese hamster ovary cells lacking an active DHFR gene are used for transfection. Five μ g of the expression plasmid pC4 is cotransfected with 0.5 μ g of the plasmid pSVneo using lipofectin (Felgner et al., *supra*). The plasmid

pSV2-neo contains a dominant selectable marker, the *neo* gene from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 1 mg/ml G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) in alpha minus MEM supplemented with 10, 25, or 50 ng/ml of methotrexate plus 1 mg/ml G418. After about 10-14 days single clones are trypsinized and then seeded in 6-well petri dishes or 10 ml flasks using different concentrations of methotrexate (50 nM, 100 nM, 200 nM, 400 nM, 800 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-well plates containing even higher concentrations of methotrexate (1 μ M, 2 μ M, 5 μ M, 10 mM, 20 mM). The same procedure is repeated until clones are obtained which grow at a concentration of 100 - 200 μ M. Expression of the desired gene product is analyzed, for instance, by SDS-PAGE and Western blot or by reversed phase HPLC analysis.

Example 4: Tissue distribution of TNF receptor mRNA expression

Northern blot analysis is carried out to examine TNFR-6 α or -6 β gene expression in human tissues, using methods described by, among others, Sambrook *et al.*, cited above. A cDNA probe containing the entire nucleotide sequence of a TNF receptor protein (SEQ ID NO:1 or 3) is labeled with ³²P using the *rediprime*TM DNA labeling system (Amersham Life Science), according to manufacturer's instructions. After labeling, the probe is purified using a CHROMA SPIN-100TM column (Clontech Laboratories, Inc.), according to manufacturer's protocol number PT1200-1. The purified labeled probe is then used to examine various human tissues for TNF receptor mRNA.

Multiple Tissue Northern (MTN) blots containing various human tissues (H) or human immune system tissues (IM) are obtained from Clontech and are examined with the labeled probe using ExpressHybTM hybridization solution (Clontech) according to manufacturer's protocol number PT1190-1. Following hybridization and washing, the blots are mounted and exposed to film at -70° C overnight, and films developed according to standard procedures.

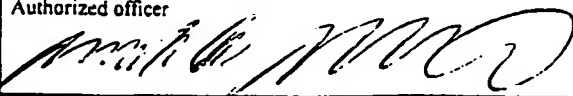
It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

The entire disclosure of all publications (including patents, patent applications, journal articles, laboratory manuals, books, or other documents) cited herein are hereby incorporated by reference.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

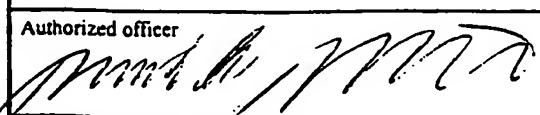
A. The indications made below relate to the microorganism referred to in the description on page <u>4</u> , line <u>13</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution American Type Culture Collection	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States of America	
Date of deposit November 22, 1996	Accession Number 97809
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

<div style="text-align: center; border-bottom: 1px solid black; margin-bottom: 5px;">For receiving Office use only</div> <div style="display: flex; align-items: center;"><input checked="checked" type="checkbox"/> This sheet was received with the international application</div> <div style="border-top: 1px solid black; padding-top: 5px;">Authorized officer </div>	<div style="text-align: center; border-bottom: 1px solid black; margin-bottom: 5px;">For International Bureau use only</div> <div style="display: flex; align-items: center;"><input type="checkbox"/> This sheet was received by the International Bureau on:</div> <div style="border-top: 1px solid black; padding-top: 5px;">Authorized officer</div>
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>4</u> , line <u>12</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution American Type Culture Collection	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States of America	
Date of deposit November 22, 1996	Accession Number 97810
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g. "Accession Number of Deposit")	

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<input type="checkbox"/> This sheet was received by the International Bureau on:
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What Is Claimed Is:

1. An isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of:
 - (a) a nucleotide sequence encoding a TNFR polypeptide having the complete amino acid sequence in SEQ ID NO:2 or 4 or as encoded by a cDNA clone contained in ATCC Deposit No. 97810 or 97809;
 - (b) a nucleotide sequence encoding a mature TNFR polypeptide having an amino acid sequence at positions 31-300 or 31-170 in SEQ ID NO:2 or 4, respectively, or as encoded by the cDNA clone contained in the ATCC Deposit No. 97810 or 97809;
 - (c) a nucleotide sequence encoding the soluble extracellular domain of a TNFR polypeptide having the amino acid sequence at positions 31-283 or 31-166 of SEQ ID NOS:2 and 4, respectively; and
 - (d) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b) or (c) above.
2. The nucleic acid molecule of claim 1 wherein said polynucleotide has a complete nucleotide sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:3.
3. The nucleic acid molecule of claim 1 wherein said polynucleotide has a nucleotide sequence which encodes a TNFR polypeptide having a complete amino acid sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.
4. The nucleic acid molecule of claim 1 wherein said polynucleotide has a nucleotide sequence encoding the mature form of a TNFR polypeptide having an amino acid sequence from about 31 to about 300 in SEQ ID NO:2 or from about 31 to about 170 in SEQ ID NO:4.

5. The nucleic acid molecule of claim 1 wherein said polynucleotide has a nucleotide sequence encoding the soluble extracellular domain of a TNFR polypeptide having the amino acid sequence from about 31 to about 283 in SEQ ID NO:2 or from about 31 to about 166 of SEQ ID NO:4.

6. An isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of:

- (a) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence of residues m-300 of SEQ ID NO:2, where n is an integer in the range of 1-49;
- (b) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence of residues n-170 of SEQ ID NO:4, where n is an integer in the range of 1-49;
- (c) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence of residues 1-y of SEQ ID NO:2, where y is an integer in the range of 193-300;
- (d) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence of residues 1-z of SEQ ID NO:4, where z is an integer in the range of 132-170; and
- (e) a nucleotide sequence encoding a polypeptide having the amino acid sequence consisting of residues m-y of SEQ ID NO:2 or n-z of SEQ ID NO:4 as m, n, y and z are defined in (a), (b), (c) and (d), above.

7. An isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of:

- (a) a nucleotide sequence encoding a polypeptide consisting of a portion of a complete TNFR amino acid sequence encoded by a cDNA clone contained in ATCC Deposit No. 97810 or 97809 wherein said portion excludes from 1 to about 48 amino acids from the amino terminus of said complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97810 and 97809;
- (b) a nucleotide sequence encoding a polypeptide consisting of a portion of a complete TNFR amino acid sequence encoded by a cDNA clone contained in ATCC Deposit No. 97810 or 97809 wherein said portion excludes from 1 to about 107 and from 1 to about 38 amino acids from the carboxy terminus of said complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97810 and 97809, respectively; and
- (c) a nucleotide sequence encoding a polypeptide consisting of a portion of a complete TNFR amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97810 or 97809, wherein said portion includes a combination of any of the amino terminal and carboxy terminal deletions for the respective clones in (a) and (b), above.

8. The nucleic acid molecule of claim 1 wherein said polynucleotide has the complete nucleotide sequence of the cDNA clone contained in ATCC Deposit No. 97810 or 97809.

9. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence encoding a TNFR polypeptide having the complete amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97810 or 97809.
10. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence encoding a mature TNFR polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97810 or 97809.
11. An isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide having a nucleotide sequence identical to a nucleotide sequence in (a), (b), (c) or (d) of claim 1 wherein said polynucleotide which hybridizes does not hybridize under stringent hybridization conditions to a polynucleotide having a nucleotide sequence consisting of only A residues or of only T residues.
12. An isolated nucleic acid molecule comprising a polynucleotide which encodes the amino acid sequence of an epitope-bearing portion of a TNFR polypeptide having an amino acid sequence in (a), (b), (c) or (d) of claim 1.
13. The isolated nucleic acid molecule of claim 12, which encodes an epitope-bearing portion of a TNFR polypeptide comprising amino acid residues selected from the group consisting of: from about Ala-31 to about Thr-46 in SEQ ID NO:2, from about Phe-57 to about Thr-117 in SEQ ID NO:2, from about Cys-132 to about Thr-175 in SEQ ID NO:2, from about Gly-185 to about Thr-194 in SEQ ID NO:2, from about Val-205 to about Asp-217 in SEQ ID NO:2, from about Pro-239 to about Leu-264 in SEQ ID NO:2, and from about Ala-283 to about Pro-298 in SEQ ID NO:2, from about Ala-31 to about Thr-46 in SEQ ID NO:4, from about Phe-57 to about Gln-80 in SEQ ID NO:4, from about Glu-86 to about His-106 in SEQ ID NO:4, from about Thr-108 to about Phe-119 in SEQ ID NO:4, from about His-129 to about Val-138 in SEQ ID NO:4, and from about Gly-142 to about Pro-166 in SEQ ID NO:4.
14. A method for making a recombinant vector comprising inserting an isolated nucleic acid molecule of claim 1 into a vector.
15. A recombinant vector produced by the method of claim 14.
16. A method of making a recombinant host cell comprising introducing the recombinant vector of claim 15 into a host cell.

17. A recombinant host cell produced by the method of claim 16.
18. A recombinant method for producing a TNFR polypeptide, comprising culturing the recombinant host cell of claim 17 under conditions such that said polypeptide is expressed and recovering said polypeptide.
19. An isolated TNFR polypeptide comprising an amino acid sequence at least 95% identical to a sequence selected from the group consisting of:
 - (a) the amino acid sequence of a full-length TNFR polypeptide having the complete amino acid sequence shown in SEQ ID NO:2 or 4, or as encoded by a cDNA clone contained in ATCC Deposit No. 97810 or 97809;
 - (b) the amino acid sequence of a mature TNFR polypeptide having the amino acid sequence at positions 31-300 in SEQ ID NO:2 or 31-170 in SEQ ID NO:4, or as encoded by a cDNA clone contained in ATCC Deposit No. 97810 or 97809; or
 - (c) the amino acid sequence of a soluble extracellular domain of a TNFR polypeptide having the amino acid sequence at positions 31 to 283 in SEQ ID NO:2 or 31 to 166 in SEQ ID NO:4, or as encoded by the cDNA clone contained in ATCC Deposit No. 97810 or 97809.
20. An isolated polypeptide comprising an epitope-bearing portion of the TNFR protein, wherein said portion is selected from the group consisting of a polypeptide comprising amino acid residues: from about Ala-31 to about Thr-46 in SEQ ID NO:2, from about Phe-57 to about Thr-117 in SEQ ID NO:2, from about Cys-132 to about Thr-175 in SEQ ID NO:2, from about Gly-185 to about Thr-194 in SEQ ID NO:2, from about Val-205 to about Asp-217 in SEQ ID NO:2, from about Pro-239 to about Leu-264 in SEQ ID NO:2, and from about Ala-283 to about Pro-298 in SEQ ID NO:2, from about Ala-31 to about Thr-46 in SEQ ID NO:4, from about Phe-57 to about Gln-80 in SEQ ID NO:4, from about Glu-86 to about His-106 in SEQ ID NO:4, from about Thr-108 to about Phe-119 in SEQ ID NO:4, from about His-129 to about Val-138 in SEQ ID NO:4, and from about Gly-142 to about Pro-166 in SEQ ID NO:4.
21. An isolated antibody that binds specifically to a TNFR polypeptide of claim 19.
22. A method of treating a patient in need of TNFR polypeptide activity comprising administering to the patient the TNFR polypeptide of claim 19.
23. A method of treating a patient in need of TNFR polypeptide activity comprising administering to the patient a nucleic acid of claim 1.

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GCTCTCCCTGCTCCAGCAAGGACCATGAGGGCGCTGGAGGGGCCAGGCCTGTCGCTGCTG
M R A L E G P G L S L I
TGCCTGGTGTGGCGCTGCCTGCCCTGCTGCCGGTGCCGGCTGTACGCGGAGTGGCAGAA
C L V L A L P A L L P V P A V R G V A E
ACACCCACCTACCCCTGGCGGGACGCAGAGACAGGGGAGCGGCTGGTGTGCGCCAGTGC
T P T Y P W R D A E T G E R L V C A Q C
CCCCCAGGCACCTTTGTGCAGCGGCCGTGCCGCCGAGACAGCCCCACGACGTGTGGCCCG
P P G T F V Q R P C R R D S P T T C G P
TGTCCACCGCGCCACTACACGCAGTTCTGGAACCTACCTGGAGCGCTGCCGCTACTGCAAC
C P P R H Y T Q F W N Y L E R C R Y C N
GTCCTCTGCGGGGAGCGTGAGGAGGAGGCACGGGCTTGCCACGCCACCCACAACCGTGCC
V L C G E R E E E A R A C H A T H N R A
TGCCGCTGCCGCACCGGCTTCTTCGCGCACGCTGGTTTCTGCTTGAGCACGCATCGTGT
C R C R T G F F A H A G F C L E H A S C
CCACCTGGTGCCGGCGTGATTGCCCCGGGCACCCCCAGCCAGAACACGCAGTGCCAGCCG
P P G A G V I A P G T P S Q N T Q C Q P
TGCCCCCAGGCACCTTCTCAGCCAGCAGCTCCAGCTCAGAGCAGTGCCAGCCCCACCGC
C P P G T F S A S S S S S E Q C Q P H R
AACTGCACGGCCCTGGGCCTGGCCCTCAATGTGCCAGGCTCTTCTCCCATGACACCCTG
N C T A L G L A L N V P G S S S H D T L
TGCACCAGCTGCACTGGCTTCCCCCTCAGCACCAGGGTACCAGGAGCTGAGGAGTGTGAG
C T S C T G F P L S T R V P G A E E C E
CGTGCCGTCATCGACTTTGTGGCTTTCAGGACATCTCCATCAAGAGGCTGCAGCGGCTG
R A V I D F V A F Q D I S I K R L Q R L
CTGCAGGCCCTCGAGGCCCGGAGGGCTGGGCTCCGACACCAAGGGCGGGCCGCGGGCC
L Q A L E A P E G W G P T P R A G R A A
TTGCAGCTGAAGCTGCGTCGGCGGCTCACGGAGCTCCTGGGGGCGCAGGACGGGGCGCTG
L Q L K L R R R L T E L L G A Q D G A L
CTGGTGCGGCTGCTGCAGGCGCTGCGCGTGCCAGGATGCCCGGGCTGGAGCGGAGCGTC
L V R L L Q A L R V A R M P G L E R S V
CGTGAGCGCTTCTCCCTGTGCACTGATCCTGGCCCCCTTATTTATTCTACATCCTTG
R E R F L P V H *
GCACCCCACTTGCACTGAAAGAGGCTTTTTTTTAAATAGAAGAAATGAGGTTTCTTAAAG
CTTATTTTATAAGCTTTTTCATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

FIG. 1

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TGGCATGTCGGTCAGGCACAGCAGGGTCCTGTGTCCGCGCTGAGCCGCGCTCTCCCTGCT
CCAGCAAGGACCATGAGGGCGCTGGAGGGGCCAGGCCTGTGCTGCTGTGCCTGGTGTG
M R A L E G P G L S L L C L V L
GCGCTGCCTGCCCTGCTGCCGGTGCCGGCTGTACGCGGAGTGGCAGAAACACCCACCTAC
A L P A L L P V P A V R G V A E T P T Y
CCCTGGCGGGACGCAGAGACAGGGGAGCGGCTGGTGTGCGCCAGTGCCCCCAGGCACC
P W R D A E T G E R L V C A Q C P P G T
TTTGTGCAGCGCCGTGCCGCCGAGACAGCCCCACGACGTGTGGCCCGTGTCCACCGCGC
F V Q R P C R R D S P T T C G P C P P R
CACTACACGCAGTTCTGGAACCTACCTGGAGCGCTGCCGCTACTGCAACGTCTCTGCGGG
H Y T Q F W N Y L E R C R Y C N V L C G
GAGCGTGAGGAGGAGGCACGGGCTTGCCAGGCCACCCACAACCGTGCCTGCCGCTGCCGC
E R E E E A R A C H A T H N R A C R C R
ACCGGCTTCTTCGCGCACGCTGGTTTCTGCTTGAGCACGCATCGTGTCCACCTGGTGCC
T G F F A H A G F C L E H A S C P P G A
GGCGTGATTGCCCCGGGTGAGAGCTGGGCGAGGGGAGGGGCCCCAGGAGTGGTGGCCGG
G V I A P G E S W A R G G A P R S G G R
AGGTGTGGCAGGGGTGAGTTGCTGGTCCCAGCCTTGACCCCTGAGCTAGGACACCAAGTT
R C G R G Q V A G P S L A P *
CCCCTGACCCTGTTCTTCCCTCCTGGCTGCAGGCACCCCCAGCCAGAACACGCAGTGCCA
GCCGTGCCCCCAGGCACCTTCTCAGCCAGCAGCTCCAGCTCAGAGCAGTGCCAGCCCCA
CCGCAACTGCACGGCCCTGGGCCTGGCCCTCAATGTGCCAGGCTCTTCCTCCCATGACAC
CCTGTGCACCAGCTGCACTGGCTTCCCCCTCAGCACCAGGGTACCAGGTGAGCCAGAGGC
CTGAGGGGGCAGCACACTGCAGGCCAGGCCCACTTGTGCCCTCACTCCTGCCCCTGACG
TGCATCTAGCCTGAGGCATGCCAGCTGGCTCTGGGAAGGGGCCACAGTGGATTTGAGGGG
TCAGGGGTCCCTCCACTAGATCCCCACCAAGTCTGCCCTCTCAGGGGTGGCTGAGAATTT
GGATCTGAGCCAGGGCACAGCCTCCCCTGGAGAGCTCTGGGAAAGTGGGCAGCAATCTCC

FIG.2A

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TAAC TGCCCGAGGGGAAGGTGGCTGGCTCCTCTGACACGGGGAAACCGAGGCCTGATGGT
AACTCTCCTAACTGCCTGAGAGGAAGGTGGCTGCCTCCTCTGACATGGGGAAACCGAGGC
CCAATGTTAACCACTGTTGAGAAGTCACAGGGGGAAGTGACCCCTTAACATCAAGTCAG
GTCCGGTCCATCTGCAGGTCCCAACTCGCCCTTCCGATGGCCAGGAGCCCAAGCCCT
TGCCTGGGCCCCCTTGCCTCTTGACGCAAGGTCCGAGTGGCCGCTCCTGCCCCCTAGGC
CTTTGCTCCAGCTCTCTGACCGAAGGCTCCTGCCCCCTTCTCCAGTCCCCATCGTTGCACT
GCCCTCTCCAGCACGGCTCACTGCACAGGGATTTCTCTCTCTGCAAACCCCCGAGTGG
GGCCCAGAAAGCAGGGTACCTGGCAGCCCCCGCCAGTGTGTGTGGGTGAAATGATCGGAC
CGCTGCCTCCCCACCCCACTGCAGGAGCTGAGGAGTGTGAGCGTGCCGTCATCGACTTTG
TGGCTTTCAGGACATCTCCATCAAGAGGAGCGGCTGCTGCAGGCCC

FIG.2B

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1	M	-	G	L	S	T	V	P	D	L	L	V	L	E	L	L	V	G	I	Y	P	S	G	V	I	G	L	V	P	H	L	G	D	R	E	-	-	TNFR1
1	M	A	P	V	A	V	W	A	A	L	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TNFR2	
1	M	G	A	G	A	T	G	R	A	M	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NGFR	
1	M	-	R	L	P	R	-	A	S	P	C	G	L	A	W	G	P	L	L	L	L	L	L	V	A	S	Q	P	Q	L	V	P	P	-	-	-	LT6R	
1	M	L	G	T	W	-	-	A	R	P	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	FAS	
1	M	-	R	V	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	CD27		
1	M	V	R	L	P	L	-	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	CD30		
1	M	G	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	CD40		
1	M	C	V	G	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	41BB		
1	M	K	S	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	OX40		
1	M	K	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	VC22		
	M	R	A	L	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	CRMB	
1	M	R	A	L	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TNFR-6a	
1	M	R	A	L	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TNFR-6b	

FIG.3A

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39	-	K	R	D	S	V	C	P	Q	G	K	G	T	Y	L	Y	N	D	C	P	G	TNFR1	
32	-	-	A	P	E	P	G	S	T	-	-	-	-	-	-	-	-	-	-	-	-	TNFR2	
34	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G A NGFR	
36	-	-	I	E	N	Q	T	T	C	W	D	Q	D	K	E	Y	A	Q	P	-	-	LIbR	
36	-	-	R	K	T	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	FAS	
29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	CD27	
22	-	-	R	P	F	E	D	T	C	H	G	N	P	S	H	Y	A	Q	P	-	-	CD30	
24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	CD40	
21	-	-	R	S	L	Q	D	P	-	-	-	-	-	-	-	-	-	-	-	-	-	41BB	
33	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	OX40	
28	-	-	P	N	G	K	C	K	D	T	E	Y	K	R	H	-	-	-	-	-	-	VC22	
26	-	-	S	N	G	K	C	K	D	N	E	Y	K	R	H	-	-	-	-	-	-	CRMB	
27	-	V	R	G	V	A	E	T	P	T	Y	P	W	R	D	A	-	E	T	G	E	R	TNFR-6a
27	-	V	R	G	V	A	E	T	P	T	Y	P	W	R	D	A	-	E	T	G	E	R	TNFR-6b

FIG.3B

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75	P	G	Q	D	T	D	C	R	-	D	C	C	E	S	G	S	-	F	T	A	S	E	N	H	L	R	H	C	L	S	C	S	G	S	K	-	C	R	K	E	M	G	TNFR1
68	T	K	T	S	D	T	V	C	-	D	C	C	-	E	D	S	T	Y	T	Q	L	W	N	W	V	P	E	C	L	S	C	T	-	R	S	S	D	Q	V	TNFR2			
60	N	Q	-	T	-	V	-	C	-	K	-	P	-	L	S	V	T	F	S	D	V	S	A	T	E	P	C	C	K	P	C	R	-	E	C	V	G	L	Q	S	NGFR		
73	S	R	S	Q	D	T	V	C	-	V	-	P	-	Q	H	N	S	Y	N	E	H	W	N	H	L	S	T	C	C	K	Q	L	C	R	-	C	D	I	V	L	G	LTbR	
76	H	-	G	D	E	P	D	C	-	D	-	P	-	I	P	G	K	Y	T	D	K	A	H	F	S	S	K	C	C	R	R	-	-	C	D	E	G	H	G	FAS			
56	H	R	K	A	-	A	Q	C	-	R	-	Q	-	-	P	G	V	S	F	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	CD27		
51	R	-	-	P	T	D	C	-	R	-	K	-	-	E	P	D	Y	Y	L	D	E	A	D	R	C	T	A	C	V	T	C	S	-	-	-	-	R	D	D	-	CD30		
52	T	E	F	T	E	-	I	C	-	L	P	C	-	-	G	E	S	E	F	L	D	W	N	R	E	T	H	C	H	Q	I	C	R	-	Q	-	-	-	K	G	CD40		
40	N	R	N	Q	-	-	-	-	-	-	S	P	C	-	-	P	P	N	S	S	A	-	G	G	Q	R	T	C	C	D	I	C	R	-	Q	-	-	-	-	-	41BB		
59	S	Q	N	T	-	V	-	C	-	R	-	P	C	-	G	P	G	F	Y	N	D	V	S	S	K	-	P	C	C	K	P	C	T	-	W	-	N	L	R	S	OX40		
60	K	T	N	T	-	-	-	-	-	-	-	-	-	-	G	S	G	T	F	T	G	R	N	H	L	P	A	C	L	S	C	N	G	R	-	C	N	Q	V	VC22			
58	K	T	N	T	-	-	-	-	-	-	-	-	-	-	A	S	D	T	F	T	S	R	N	H	L	P	A	C	L	S	C	N	G	R	-	C	D	S	N	Q	V	CRMB	
63	R	R	D	S	P	T	T	C	-	G	P	C	-	P	P	R	H	Y	T	Q	F	W	N	Y	L	E	R	C	R	Y	C	N	V	L	C	G	E	R	E	E	TNFR-6a		
63	R	R	D	S	P	T	T	C	-	G	P	C	-	P	P	R	H	Y	T	Q	F	W	N	Y	L	E	R	C	R	Y	C	N	V	L	C	G	E	R	E	E	TNFR-6b		

FIG.3C

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111	-	Q	V	E	I	S	S	C	T	V	D	R	D	T	V	C	G	C	R	K	N	Q	Y	R	H	Y	W	S	E	N	L	F	Q	C	F	N	C	S	L	-	TNFR1		
106	-	-	E	T	Q	A	-	-	C	T	R	E	Q	N	R	I	C	T	C	R	P	G	W	Y	C	A	L	S	K	Q	E	-	-	G	C	R	L	C	A	P	L	TNFR2	
95	M	S	A	P	-	-	Q	-	C	V	E	A	D	D	A	V	C	R	C	A	Y	G	Y	-	Q	D	E	T	T	G	-	-	R	C	E	A	C	R	V	O	NGFR		
110	F	E	V	A	P	-	-	-	C	T	S	D	R	K	A	E	C	R	C	Q	P	G	S	V	Y	L	D	N	E	-	-	-	-	C	V	H	C	E	E	L	TbR		
113	L	E	V	E	I	N	-	-	C	T	R	T	Q	N	T	K	C	R	C	K	P	N	-	-	-	-	-	-	-	-	-	-	-	-	F	F	C	N	-	-	FAS		
74	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D	H	T	R	P	-	-	-	-	H	C	E	S	C	R	H	-	CD27		
92	L	V	E	K	T	P	-	-	C	A	W	N	S	S	R	V	C	E	C	R	P	G	M	F	C	S	T	S	V	N	-	-	-	S	C	A	R	C	F	H	CD30		
89	L	R	V	Q	Q	K	-	-	G	T	S	E	T	D	T	I	C	T	C	E	E	G	W	H	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	CD40	
71	V	F	R	T	R	K	E	-	C	S	S	T	S	H	A	E	C	C	T	P	G	F	H	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	41BB	
92	G	S	E	R	K	Q	L	-	C	T	A	T	Q	D	T	V	C	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	OX40	
96	-	-	E	T	R	S	-	-	C	N	T	T	H	H	R	I	C	E	C	S	P	G	Y	Y	C	L	L	K	G	S	-	-	-	G	C	K	A	C	V	S	Q	VC22	
96	-	-	E	T	R	S	-	-	C	N	T	T	H	H	R	I	C	E	C	A	P	G	Y	Y	C	L	L	K	G	S	-	-	-	G	C	K	A	C	V	S	Q	CRMB	
101	-	-	E	A	R	A	-	-	C	H	A	T	H	N	R	A	C	R	C	R	T	G	F	F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TNFR-6a
101	-	-	E	A	R	A	-	-	C	H	A	T	H	N	R	A	C	R	C	R	T	G	F	F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TNFR-6b

FIG.3D

13/22

264	T	K	P	L	A	P	N	P	S	F	S	P	T	P	G	F	T	P	T	L	G	F	S	P	V	P	S	S	T	F	T	S	S	T	Y	T	P	G	D	TNFR1		
294	-	-	-	-	-	-	-	-	L	Q	R	E	A	K	V	P	H	L	P	A	-	D	K	A	R	G	T	Q	G	P	E	Q	Q	H	L	L	I	T	A	-	TNFR2	
281	-	-	-	-	-	-	-	-	-	N	K	Q	G	A	N	S	R	P	V	-	N	Q	T	P	P	P	E	G	E	K	L	H	S	D	S	G	I	S	V	D	NGFR	
262	-	-	-	-	-	-	-	-	-	P	E	G	E	S	P	P	C	P	A	-	P	R	A	D	P	H	F	F	D	L	A	E	P	L	-	-	-	-	-	LTbR		
212	-	-	-	-	-	-	-	-	S	P	T	L	N	P	E	-	-	T	V	A	I	N	L	S	D	V	D	L	S	K	Y	I	T	-	-	-	-	-	-	FAS		
169	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	CD27			
322	T	T	F	E	A	P	P	L	G	T	Q	P	D	C	N	P	T	P	E	-	N	G	E	A	P	A	S	T	S	P	T	Q	S	L	L	V	D	S	Q	A	CD30	
222	-	-	-	-	-	-	-	-	-	P	T	N	K	A	P	H	P	K	Q	E	-	P	Q	-	E	I	N	F	F	D	-	-	-	-	-	-	-	-	-	-	CD40	
193	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	41BB		
198	-	-	-	-	-	-	-	-	-	-	T	S	Q	G	P	S	T	R	P	V	-	E	-	-	-	V	P	G	G	R	A	V	A	I	L	G	L	G	L	-	CK40	
255	-	-	-	-	-	-	-	-	-	L	N	F	E	I	K	C	N	N	-	-	-	-	-	-	K	G	S	-	S	F	K	Q	-	-	-	-	-	-	-	-	VC22	
255	-	-	-	-	-	-	-	-	-	L	N	F	E	I	K	C	N	N	-	-	-	-	-	-	K	D	S	Y	S	S	S	K	Q	-	-	-	-	-	-	-	CRMB	
229	-	-	-	-	-	-	-	-	-	L	Q	R	L	L	Q	A	L	E	A	P	E	-	G	W	-	G	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TNFR-6a
143	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	S	W	A	R	G	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TNFR-6b

FIG.3J

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305	C P N F A . A	P R R E V A P P Y Q G A D P I L A T A - - - - -	-	-	-	-	-	-	-	TNFR1
325	- - - - -	S S S S S L E S A S A L - - - - -	-	-	-	-	-	-	-	TNFR2
312	S Q S L H D Q	Q P H T Q T A S G Q A L K - - - - -	G	D	G	G	L	Y	S	NGFR
286	- - - - -	M S G D L S P S P A G P P T - - - - -	-	-	-	-	-	-	-	Lt6R
236	- - - - -	- - - - - U A G V - - - - -	-	-	-	-	-	-	-	FAS
171	A R T L S T H W P P Q R S L C S D F I - - - - -	R I L V I F S G M F L V F T L A C D 27	-	-	-	-	-	-	-	CD27
361	S K T L P I P T S A P V A L S S T G K P V L D A G P V L F W V I L V L V V V G C D 30	-	-	-	-	-	-	-	-	CD30
241	- - - - -	D L P G S N T A A P V - - - - -	-	-	-	-	-	-	-	CD40
193	- - - - -	- - - A L T A L L F L - - - - -	-	-	-	-	-	-	-	CD40
224	- - - - -	- - - - - L F F L T L R F S V V K R 41BB	-	-	-	-	-	-	-	41BB
274	- - - - -	- - - A K N D D G M - - - - -	G	P	L	A	I	L	L	OX40
276	- - - - -	- - - T K N D D S I - - - - -	-	-	-	-	-	-	-	VC22
										CRMB

FIG. 3G

246	- - - - -	P R A G R A A L Q L K - - - - -	-	-	-	-	-	-	-	TNFR-6a
151	- - - - -	P R S G - - - - -	-	-	-	-	-	-	-	TNFR-6b

FIG. 3K

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[illegible]

FIG. 3L

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365	N	V	P	P	L	R	W	K	E	F	V	R	R	L	G	L	S	D	H	E	I	D	R	L	E	L	Q	N	G	R	C	L	R	E	A	Q	Y	S	M	L	TNFR1	
372	P	G	G	H	G	T	Q	V	N	V	T	C	I	V	N	V	C	S	S	D	H	-	S	S	Q	C	S	S	Q	A	S	S	T	M	G	D	T	-	-	TNFR2		
355	A	G	D	T	W	R	H	L	A	G	E	L	G	I	H	V	E	H	I	D	S	F	T	H	E	A	C	P	V	R	-	-	-	-	-	A	L	L	NGFR			
327	P	G	E	H	G	Q	V	A	H	G	A	N	G	I	H	V	T	G	S	V	T	V	T	G	N	I	Y	I	Y	N	G	P	V	L	G	G	T	-	-	LTbR		
246	-	-	-	-	-	-	-	-	K	G	F	V	R	K	N	G	V	N	E	A	K	I	D	E	I	K	N	D	N	V	Q	D	T	A	E	Q	K	V	Q	L	L	FAS
214	-	-	-	-	-	-	-	-	R	K	Y	R	S	N	K	G	E	S	P	V	E	P	C	R	Y	S	C	P	R	-	-	-	-	-	-	-	-	-	-	CD27		
441	R	R	S	S	T	Q	L	R	S	G	A	S	V	T	E	P	V	A	E	E	R	G	L	M	S	Q	P	L	M	E	T	C	H	S	V	G	A	A	Y	L	CD30	
256	-	-	-	-	-	-	-	-	H	G	C	Q	P	V	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	CD40			
222	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	41BB				
245	-	-	-	-	-	-	-	-	R	L	P	P	D	-	A	H	K	P	P	G	G	S	F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	OX40	
303	A	Q	D	Y	E	T	D	T	I	S	Y	R	V	G	N	V	L	D	D	D	S	H	M	P	G	S	C	N	I	H	K	-	-	-	-	-	-	-	-	-	VC22	
311	T	Q	D	Y	E	T	D	T	I	S	Y	H	V	G	N	V	L	D	V	D	S	H	M	P	G	R	C	D	T	H	K	-	-	-	-	-	-	-	-	CRMB		
282	-	-	-	-	-	-	-	-	-	-	-	V	A	R	M	P	G	L	E	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TNFR-6a			
155	-	-	-	-	-	-	-	-	-	-	-	G	R	R	C	G	R	G	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	TNFR-6b			

FIG.3M

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[illegible]

FIG. 3N

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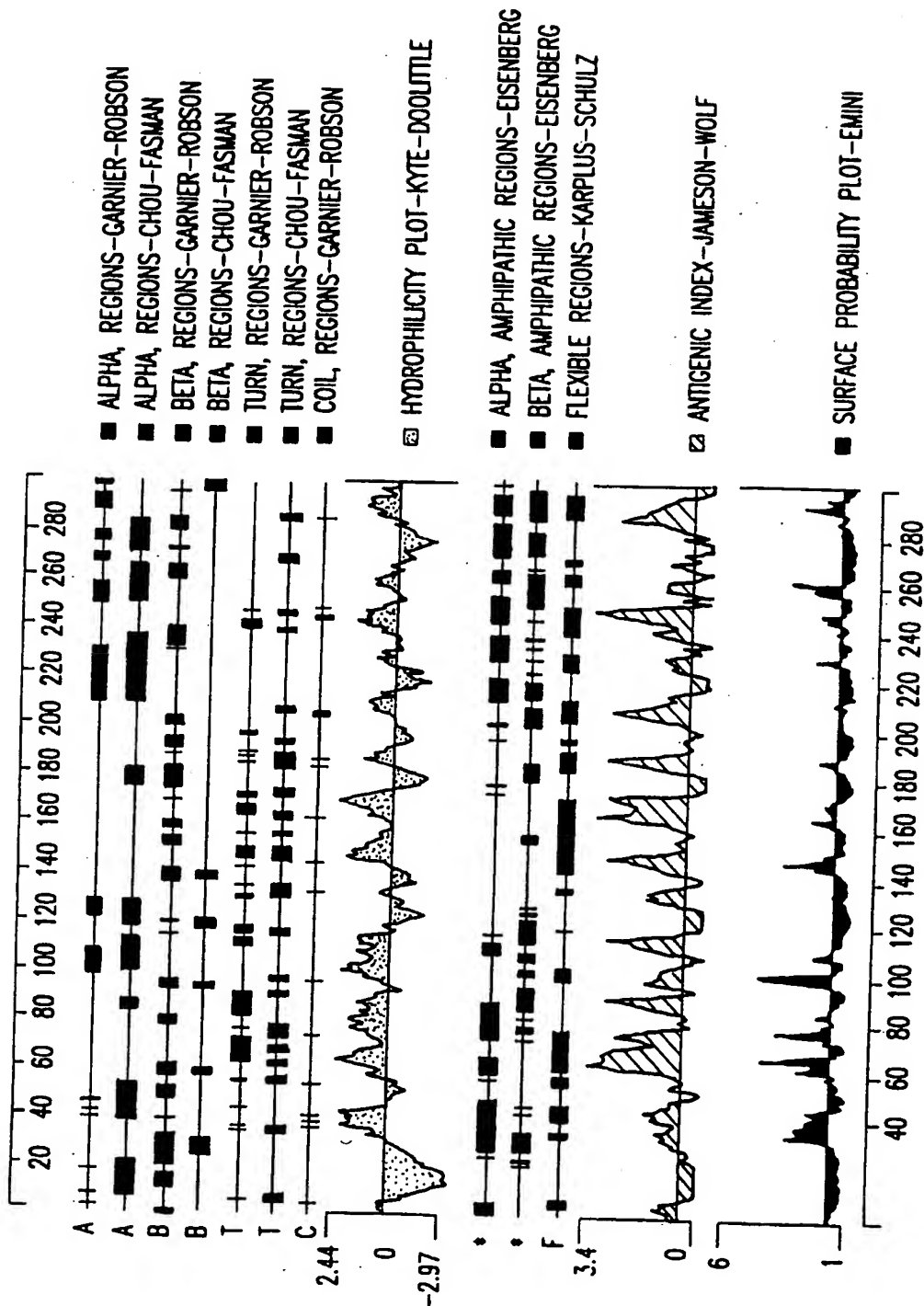


FIG.4

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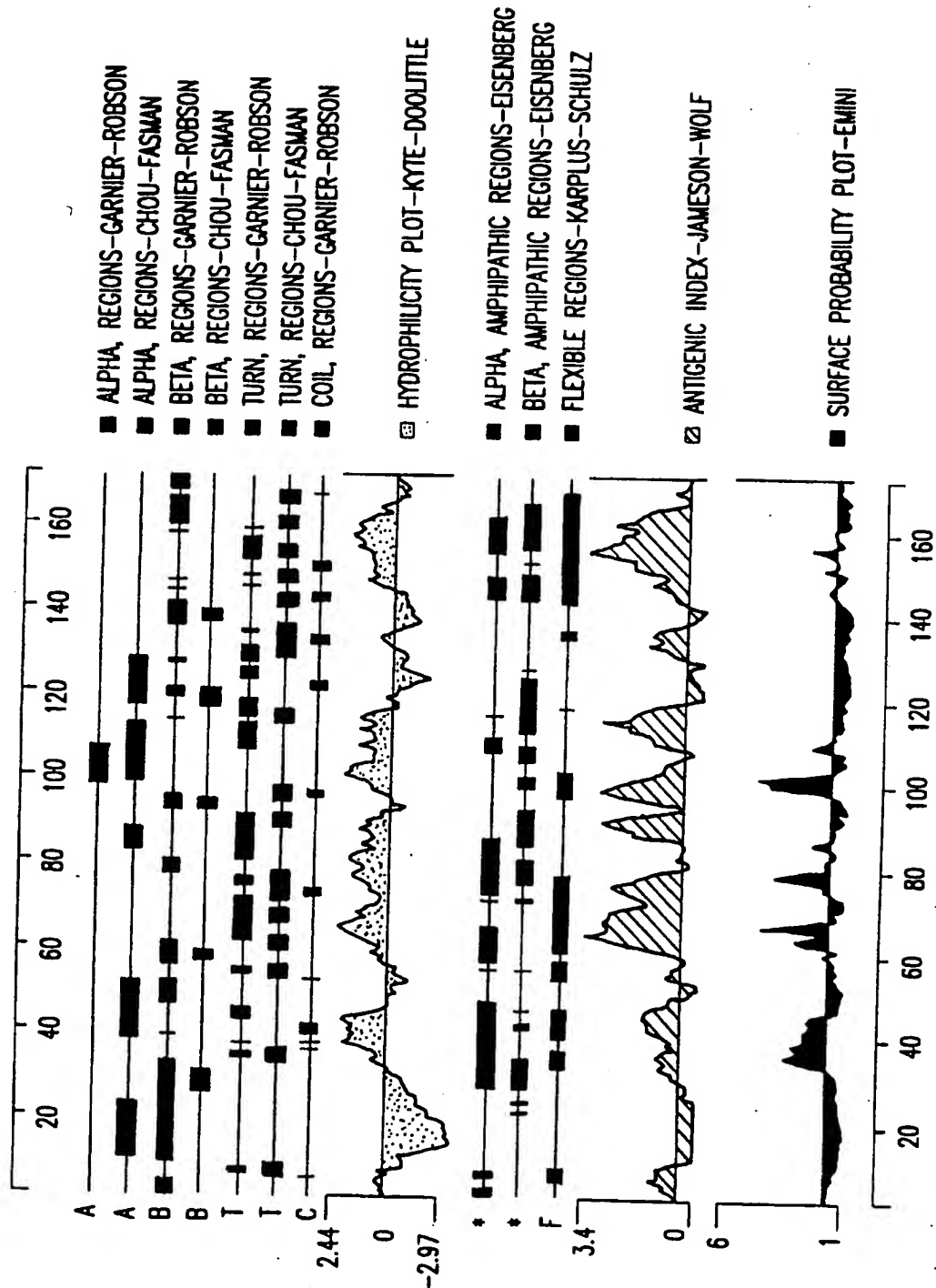


FIG.5

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HELDI06R

GGCAGAGCA GGGTCCTGTN TCCGCCCTGA GCCGCGCTCT NCCTGCTCCA GCAAGGACCA
TGAGGGCGCT GGAGGGGCCA GGCCTGTCGC TGCTGTGCCT GGTGTTGGCG CTGCCTGCCC
TGCTGCCGGT GCCGGCTGTA CGCGGAGTGG CAGAAACACN NACNTACCCC TGGCGGGACG
NAGAGACAGG GGAGCGGCTG GTGTNTNCCC ANTGCCCCC AGGCACCTTT NTGCAGCGGC
CGTGCCGNCG AGACAGCCCC ACGACGTGTG GCGCGTNTCC ACCGCGCCAC TACACGCATT
CTGGAACACT CTGGAGCGCT GNCGTTACTN CAACGTCCTC TCGGGGAGC GTNAGGAGGA
GGCAGGGTT TNCCACGNCA ACCACAACCG NGGNTTACCG TNGCCGNACC GGTTCCTCG
NGGCAAGTTG GTTTTTNNTT TGGAGNAAGG ATTCGTGTTN CAATTNATTG ACGNAGTGAT
TNNCNCGGG AACTNAAA

HCEOW38R

CGCAACTGCA CGGCCCTGGG ACTGGCCCTC AATGTGCCAG GNTCTTCCTC CCATGACACC
CTGTGCACCA GCTGCACTGG CTTCCCCCTC AGCACCAGGG TACCANGAGC TGAGGAGTGT
GAGCNTGCCG TCATCGACTT TTTGGCTTTC CAGGACATCT CCATCAAGAG GCTGCAGCGG
CTGCTCANGC C

FIG.6